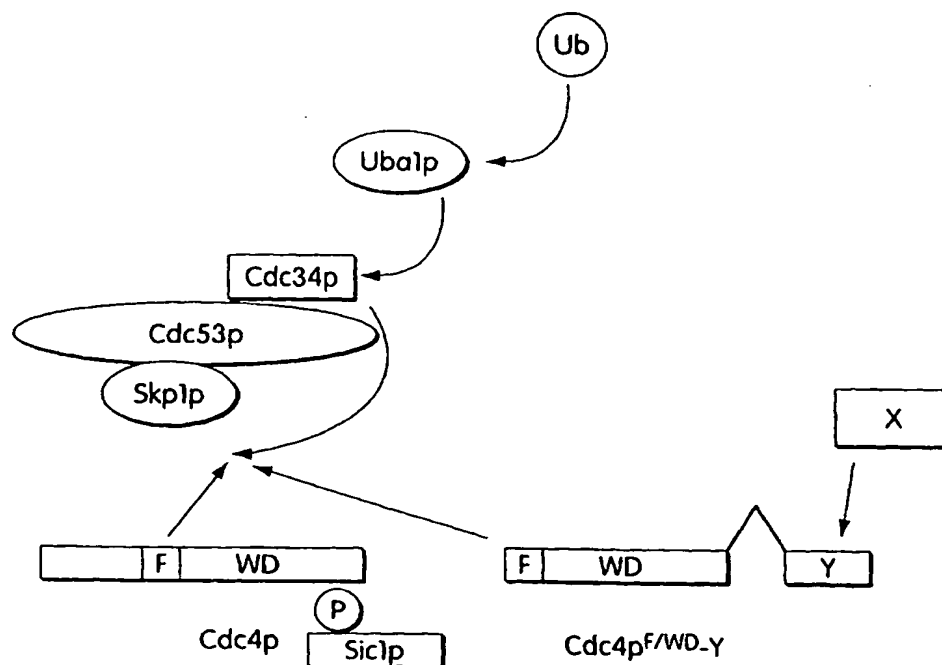




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : C12N 15/00, 15/62, 15/12, 15/37, 15/52, 5/10 // C07K 14/00		A2	(11) International Publication Number: <b>WO 00/22110</b>
			(43) International Publication Date: 20 April 2000 (20.04.00)
(21) International Application Number: PCT/US99/23705 (22) International Filing Date: 8 October 1999 (08.10.99) (30) Priority Data: 60/103,787 9 October 1998 (09.10.98) US (71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). (72) Inventors: ZHOU, Pengbo; 34 Hammond Pond Parkway #3, Chestnut Hill, MA 02467 (US). HOWLEY, Peter; 5 Hundreds Circle, Wellesley, MA 02181 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	

(54) Title: TARGETED PROTEOLYSIS BY RECRUITMENT TO UBIQUITIN PROTEIN LIGASES



## (57) Abstract

The present invention relates to methods and reagents for targeting proteolysis of a polypeptide by cis or trans association with a ubiquitin protein ligase, and further provides methods and reagents for inhibiting the ubiquitination and proteolysis of cellular proteins which are recognized by a ubiquitin protein ligase.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

## **Targeted Proteolysis by Recruitment to Ubiquitin Protein Ligases**

### **1. Background of The Invention**

Many diseases and pathological conditions involve the inappropriate expression or undesirable activity of one or more cellular genes. Examples include cancer, in which one or more cellular oncogenes become activated and results in the unchecked progression of cell cycle processes. Furthermore, many human genetic diseases, such as Huntington's disease and certain prion conditions, result from the inappropriate activity of a polypeptide as opposed to the loss of its function. Drug therapy strategies for these disorders have frequently employed molecular antagonists which target the polypeptide product of the disease gene(s). Additionally, infectious diseases such as HIV have been successfully treated with molecular antagonists targeted to specific essential retroviral gene(s). The discovery or rational design of such disease gene or pathogen gene antagonists is often difficult and time-consuming. Furthermore, such molecular antagonists frequently have unanticipated effects on other cellular activities which were not targeted for therapeutic intervention. Therefore a systematic means for degrading one or more target polypeptides which cause or contribute to a disease or condition would allow the rapid development of suitable antagonist therapeutic agents once a disease-causing polypeptide or pathogen gen function is identified.

The ability to systematically antagonize specific target polypeptide activities would further allow for the general elucidation and control of the essential polypeptide-mediated functions of a host cell. Indeed, the field of genetics is essentially an approach to understanding biological processes through the systematic elimination of cellular polypeptide functions. Historically, the genetic approach has involved the development of "screens" for mutations in genes which affect a specific phenotypic trait of an organism. The great advantage of this approach has been that no prior knowledge of the molecular nature of the genes involved is required because the "screen" identifies the affected genes by marking them with mutations. The mutation involved is frequently a change in the gene's sequence which results in a loss-of-function of the encoded gene product. Unfortunately the genetic approach has many limitations. Indeed the study of essential genes, required for cell viability, is exceedingly difficult using a purely genetic approach. The implementation of "reverse genetics" in yeast (see e.g. Winston et al. (1983) *Methods Enzymol* 101: 211-28) and, later, in mammals (see e.g. Capecchi (1989) *Science* 244: 1288-92), has allowed the positive identification of a gene as essential through the

**SUBSTITUTE SHEET (RULE 26)**

inability to recover viable yeast haploid gene "knockout" spores or homozygous recessive "knockout" mice. Nevertheless, the exact biological processes in which the essential gene is involved are difficult to determine due to the inability to isolate and/or study the doomed knockout yeast spore or the inviable homozygous mouse zygote. Thus the downstream effects on specific aspects of cell function following removal of the essential gene product cannot be readily determined using these traditional "knockout" studies. Furthermore, while traditional gene "knockout" experiments may be useful in demonstrating that a given gene is essential for the life of the organism, they provide neither a convenient method for determining how rapidly cellular function ceases nor what cellular processes immediately follow the removal of the gene product, nor further do they allow for the facile determination of the rate at which so-called "second-site suppressing" mutations can arise which restore cell viability following the removal of the essential gene. These considerations are important in the selection of targets for the rational design of, for example, antibiotic or chemotherapeutic pharmaceutical agents.

The functional characterization of a specific cellular protein often relies on experimentally altering the levels of the protein of interest, and studying the biological consequences of this manipulation. Gene knockout, ribozyme, anti-sense or RNA-mediated interference (RNAI) technologies are frequently used in different eukaryotic organisms to either eliminate or reduce the levels of a cellular protein. Antisense oligonucleotides and designer ribozymes provide a means for the systematic removal of a specific gene product without the prior bioengineering of the cellular target. (see, e.g. Hargest and Williamson (1996) *Gene Ther.* 3: 97-102; Lipkowitz et al. (1996) *Am J Kidney Dis* 28: 475-92; and Bennett and Schwartz (1995) *Circulation* 92: 1981-93); however the successful employment of such nucleic acid antagonists has met with many difficulties. Furthermore, where the immediate removal of existing gene product is desired, antisense and ribozyme gene antagonists are relatively inefficient since existing gene products are removed only at the rate of their natural turnover.

A systematic, rational means for antagonizing the function of a specific cellular or pathogen gene would provide a powerful strategy for dealing with certain disease conditions as well as providing a means to determine and control the essential biological functions of a host cell.

A number of methods for the directed inactivation of a specific target polypeptide functions in a host eucaryotic cell have been developed. For example, in an attempt to provide for a systematic means of deriving temperature -sensitive conditional alleles of a given gene target, Dohmen et al. have devised a temperature-sensitive "degron" cassette that can be



appended to any gene of interest and used to render it thermosensitive (Dohmen et al. (1994) Science 263: 1273-6). The generality with which this thermosensitive degron can be successfully applied to any target polypeptide has yet to be determined and the necessity of relying upon thermal induction for the resulting system is a major drawback. Indeed, eucaryotic cells experience a transient heat-shock response which can have profound effects on some cellular processes such as transcription. Furthermore, the requirement for induction by heat shock precludes useful application to mammalian transgenic animal systems. Another example of a system for the controlled removal of a specific gene product is the use of the ubiquitin N-end rule proteolytic system to rapidly and inducibly degrade a specific target which has been engineered to carry a predisposing non-methionine amino terminal amino acid (Moqtaderi, et al. (1996) Nature 383: 188-91). This system can be made inducible by engineering the host cell to express a key component(s) of the N-end rule proteolytic system under the control of a suitable promoter, such as a copper-inducible promoter. Still other systems have been developed for the controlled removal of a specific host gene. Notably the Cre/lox system (see e.g. Sauer (1998) Methods 14: 381-92) allows for the inducible deletion of a specific target gene through the action of the Cre site-specific DNA recombinase. Using this system, genetic switches can be designed to ablate a specific target gene in a specific tissue and at a specific time during development. One shortcoming of this method is that, following recombinational deletion of the targeted gene from the chromosome, the remaining mRNA and polypeptide products of the gene may only slowly be titrated out of the host cell through consecutive mitotic cell divisions and/or the eventual turnover of the mRNA and polypeptide by cellular ribonucleases and proteases. Thus it would be desirable to have a more rapid means for directly inactivating specific target genes in a host eucaryotic cell. A principal disadvantage of all of these systems is that they require the specific bioengineering of the target cell or host to potentiate the removal of the targeted gene function. In particular, these methods require the molecular modification of the target polypeptide-encoding gene and so it is difficult to employ them in the alteration of endogenous cellular protein levels. Therefore these techniques, while potentially useful in the identification and study of gene function in a model organism, are unlikely to be applicable to the therapeutic treatment of specific human disease conditions.

The steady state level of a cellular protein is reached when the balance is achieved between the rates of its synthesis and degradation. Altering the amount of a cellular protein has been a fundamental approach in understanding its normal cellular function. Many important technologies have been developed to manipulate cellular protein levels. Gene transfer and

transgenic technologies in tissue culture or in animals are frequently used to overproduce proteins, whereas gene knockout, ribozyme or RNAI have been used to either eliminate or reduce expression of specific proteins. Although complete elimination of a cellular protein often allows the determination of the cellular functions of a protein, there are instances where the reduction in the level of a specific gene product may be desirable in order to assess the function of a specific protein in a specific process. Furthermore, complete gene knockout is sometimes not desirable, in situations in which the deletion of a particular gene has pleiotropic effects that may overshadow specific cellular functions.

Ubiquitin dependent proteolysis is a major catabolic pathway utilized by eukaryotic cells for the degradation of cellular proteins. Protein ubiquitination is catalyzed by the concerted actions of three classes of enzymes; the E1 ubiquitin-activating enzymes, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin protein ligases (reviewed in Hochstrasser (1996) *Annu Rev. Genet* 30: 405-39). While E1 and E2 are primarily involved in the activation and transfer of ubiquitin, the substrate specificity of the ubiquitin pathway is conferred by the E3 ubiquitin protein ligases. Recent studies have revealed distinct E3 components and ubiquitination machineries that operate at various cellular processes to target distinct substrates for degradation.

For example, the SCF (Skp1, Cullin and F-box-containing proteins) ubiquitin protein ligases function to promote cell cycle transitions by targeting multiple cell cycle regulators for ubiquitin-dependent proteolysis (reviewed in King et al. (1996) *Science* 274: 1652-9; Patton et al. (1998) *Genes Dev* 12: 692-705; Koepp et al. (1999) *Cell* 97: 431-4; and references therein). Among the subunits of the SCF complexes, Skp1 and cullin are thought to form a stable core complex that is shared by different F-box-containing proteins, and interacts with E2 through the cullin subunit. Multiple F-box proteins exist that serve as receptors for recognition and recruitment of various target polypeptides to the core SCF for ubiquitination. Different F-box proteins share the common F-box domain for Skp1 binding, but utilize additional modular protein-protein interaction domains, such as WD40 or leucine-rich repeats (LRR) for binding distinct classes of substrates. Recently, a fourth subunit of SCFs, Rbx-1/Roc1, was identified that interacts with F-box proteins, and is thought to stimulate interaction between the Cdc34 ubiquitin-conjugating enzyme and SCF ubiquitin-protein ligases (Kamura et al. (1999) *Science* 284: 657-61; Skowyra (1999) *Science* 284: 662-5; Ohta et al. (1999) *Mol Cell* 3: 535-41; Tan et al. (1999) *Mol Cell* 3: 527-33). Our recent studies indicated that the yeast F-box-containing proteins, such as Cdc4p, is itself a target of the SCF-mediated ubiquitination

and degradation (Zhou and Howley (1998) Mol Cell 2: 571-80). This suggests that the intact SCF complexes may be transient in nature, and that the proteolysis of the F-box proteins is essential for maintaining the SCF proteolytic activities by permitting the exchange of the substrate recognition components.

Transit through the G1/S boundary and initiation of DNA synthesis in the yeast *Saccharomyces cerevisiae* require ubiquitin-dependent proteolysis of the CDK inhibitor Sic1p by an SCF ubiquitin ligase (reviewed in (Deshaies (1997) Curr Opin Genet Dev 7: 7-16; King et al. (1996) Science 274: 1652-9; and references therein). Genetic analyses have identified four proteins, Cdc34p, Cdc4p, Cdc53p, and Sklp, whose functions are required for Sic1p ubiquitination (Bai et al. (1996) Cell 86: 263-74; Mathias et al. (1996) Mol Cell Biol 16: 6634-43; Schwob et al. (1994) Cell 79: 233-44). Recently, the Sic1p ubiquitination pathway was biochemically defined by *in vitro* reconstitution using recombinant proteins (Feldman et al. (1997) Cell 91: 221-30; Skowyra et al. (1997) Cell 91: 209-19). The E3 activity is conferred by a multiprotein complex, designated SCF<sup>Cdc4p</sup>, consisting of Sklp, Cdc53p and Cdc4p. In association with the Cdc34p ubiquitin conjugating enzyme, SCF<sup>Cdc4p</sup> promotes Sic1p ubiquitination *in vitro* in the presence of the E1 ubiquitin activating enzyme and ubiquitin. Importantly, phosphorylation of Sic1p by Cdc28p/G1 cyclin dependent kinase is a prerequisite for Sic1p recognition by Cdc4p (Feldman et al. (1997) Cell 91: 221-30; Schneider et al. (1996) Science 272: 560-2; Schwob et al. (1994) Cell 79: 233-44; Skowyra et al. (1997) Cell 91: 209-19; Verma et al. (1997) Science 278: 455-60). Although the *in vitro* reconstitution experiments have defined the essential ubiquitin enzyme components and the biochemical mechanisms for the Cdc34p/SCF<sup>Cdc4p</sup> ubiquitination pathway, the question remains as to how the individual components collaborate *in vivo* to deliver ubiquitin to SCF<sup>Cdc4p</sup> substrates, and how the activity of the SCF<sup>Cdc4p</sup> ubiquitin protein ligase complex is controlled through the various cell cycle transitions.

Mutations of the Cdc34p/SCF<sup>Cdc4p</sup> pathway components affect the stability of several regulators of cell growth in addition to Sic1p, including the Cdk inhibitor Far1p (Henchoz et al. (1997) Genes Dev 11: 3046-60; McKinney et al. (1993) Genes Dev 7: 833-43), the p58<sup>CtIF13</sup> subunit of the Cbf3 kinetochore assembly complex (Kaplan et al. (1997) Cell 91: 491-500), the DNA replication protein Cdc6p (Drury et al. (1997) EMBO J 16: 5966-76; Piatti et al. (1996) Genes Dev 10: 1516-31), and the GCN4p transcription factor (Kornitzer et al. (1994) EMBO J 13: 6021-30). The Cdc34p/SCF<sup>Cdc4p</sup> proteolytic activity has also been implicated in phases of cell cycle other than G1/S (King et al. (1996) Science 274: 1652-9), as

deletion of *SIC1* allows *cdc4<sup>ts</sup>*, *cdc34<sup>ts</sup>*, *cdc53<sup>ts</sup>* and *skp1<sup>ts</sup>* cells to pass through the G1/S boundary, but they become arrested at G2-M (Bai et al. (1996) Cell 86: 263-74; Schwob et al. (1994) Cell 79: 233-44). Therefore, the Cdc34p/SCF<sup>Cdc4p</sup> pathway must be active at different stages of the cell cycle to mediate the degradation of a variety of substrates (Deshaies (1997) Curr Opin Genet Dev 7: 7-16; King et al. (1996) Science 274: 1652-9).

Two additional SCF complexes, SCF<sup>Grr1p</sup> and SCF<sup>Met30p</sup>, have also been identified in *S. cerevisiae* and are implicated in the degradation of G1-specific cyclins or regulators of sulfur metabolism, respectively (Patton et al. (1998) Genes Dev 12: 692-705; Skowyra et al. (1997) Cell 91: 209-19). The three SCFs share in common the Cdc53p and Skp1p components, and differ in the F-box-containing subunits, Cdc4p or Grr1p or Met30p. It is these different F-box-containing proteins that serve as the substrate recognition subunits. It is currently unclear whether the individual intact SCFs are somehow regulated within cells, or whether there is an equilibrium involving, the transient assembly of SCFs with the individual F-box subunit recognition component. Here we report that the F-box-containing subunits of SCFs are intrinsically short-lived proteins. The SCF<sup>Cdc4p</sup> machinery mediates the autoubiquitination and degradation of the F-box-containing Cdc4p, the substrate recognition component of SCF<sup>Cdc4p</sup>. Our studies suggest a novel mechanism for the rapid assembly and disassembly of distinct SCF complexes through auto-ubiquitination of the F-box-containing SCF components, which allows the cell to respond in a timely manner to different cell cycle or environmental signals to target a variety of specific cellular proteins for proteolysis.

The SCF ubiquitin ligases are only one group of known ubiquitin ligases. Other groups of structurally related ubiquitin ligases include the HECT domain ubiquitin ligases which include mammalian E6-AP and Nedd-4, and yeast RSP5 (see Huibegtsse et al. (1995) Proc Natl Acad Sci USA 92: 2563-7; Wang et al. (1999) Mol Cell Biol 19: 342-52) as well as the more recently discovered HECT domain ubiquitin ligase proteins such as mammalian Smurf1 (Zhu, et al. (1999) Nature 400: 687-93), yeast TOM1 (Saleh et al. (1998) J Mol Biol 282: 933-46; Utsugi et al. (1999) Gene 234: 285-95), and human EDD (Callaghan et al. (1999) Oncogene 17: 3479-91). The HECT domain ubiquitin ligases effect ubiquitin conjugation, and thereby activate targeted proteolysis, of a number of polypeptide targets such as the *Schizosaccharomyces pombe* mitotic activating tyrosine phosphatase cdc25p (Nefsky and Beach (1996) EMBO J 15: 1301-12) and the SMADs, a group of proteins which control embryonic development and a wide variety of cellular responses to TGF-beta signals (Zhu et al. (1999) Nature 400: 687-93). While many targets of HECT-mediated ubiquitin-dependent proteolysis interact directly with their target

protein, others interact through a third "adaptor" polypeptide which itself is not targeted for ubiquitination. For example, Grb10 remains unubiquitinated following its interaction with Nedd4 HECT ubiquitin ligase, and may serve to target other proteins, such as tyrosine kinase receptors, for ubiquitination (Morriane et al. (1999) J Biol Chem 274L 24094-9).

A third group of ubiquitin protein ligases are those involved in the degradation of polypeptides with particular amino-terminal amino acid residues. These include the yeast and mammalian Ubr1p N-end rule ubiquitin ligases which bind to destabilizing target polypeptide N-terminal residues and facilitate ubiquitination of the bound target (Kwon et al. (1998) Proc Natl Acad Sci USA 95: 7893-903; Varshavsky (1996) Proc Natl Acad Sci USA 93: 12142-9). This group of ubiquitin protein ligases likely function to control the intracellular levels of polypeptides which have undergone an endoproteolytic processing event which removes the normal stabilizing amino-terminal methionine residue. Particular classes of amino acid residues such as the basic residues (arginine, lysine and histidine) or the bulky hydrophobic residues (phenylalanine, leucine, tryptophan, tyrosine, and isoleucine), when exposed at the amino-terminus of the resulting processed protein, result in recruitment of Ubr1p-type ubiquitin protein ligases which effect ubiquitination of the target, resulting eventually in the proteasome-dependent destruction of the target polypeptide.

It is an object of the instant invention to exploit the ubiquitin protein ligases for experimental and therapeutic purposes by providing modified ubiquitin protein ligase polypeptides which recruit target polypeptides for ubiquitin conjugation and ubiquitin-dependent proteolytic degradation by a cellular proteasome. Such modified ubiquitin protein ligases allow for the specific and regulated destruction of any target polypeptide which is cloned or for which an interaction domain is available. It is a further an object of the invention to provide inhibitors of ubiquitin protein ligases which prevent their interaction and/or ubiquitination of a target protein which is present in a host cell. These ubiquitin protein ligase inhibitors allow for the specific blocking of ubiquitin-mediated degradation of a target protein and thereby stabilize the level of the target protein in the host cell. Therefore, the invention generally provides methods and compositions to stimulate a ubiquitin protein ligase activity on a target polypeptide, or to repress a ubiquitin protein ligase activity on a target polypeptide.

## **2. Summary of the Invention**

The invention generally provides methods for increasing or decreasing a ubiquitin protein ligase ubiquitination activity on a target polypeptide. In general, increased ubiquitination

of a target polypeptide is effected by cis or trans targeting the target polypeptide to a ubiquitin protein ligase while decreased ubiquitination of the target polypeptide is effected by contacting the target polypeptide with a target polypeptide ubiquitination inhibitor of the invention.

In a preferred embodiment, the invention provides a method for targeting degradation of a polypeptide in vivo by providing a ubiquitin protein ligase polypeptide that encodes a ubiquitin conjugation activity, functionally linking the ubiquitin protein ligase polypeptide to a target polypeptide interaction domain that provides a target polypeptide recruitment activity, and expressing the ubiquitin protein ligase polypeptide-target polypeptide interaction domain hybrid in a cell. The hybrid protein recruits the target polypeptide through the target polypeptide interaction domain and ubiquitinates the target polypeptide through the ubiquitin protein ligase domain. The resulting ubiquitinated target polypeptide is typically further subject to ubiquitin-dependent proteolysis by a cellular proteasome.

In certain embodiments, the ubiquitin protein ligase polypeptide is an E3 ubiquitin protein ligase polypeptide, preferably an SCF polypeptide, a HECT polypeptide, or a UBR1 polypeptide. In preferred embodiments, the E3 ubiquitin protein ligase polypeptide is an F-box polypeptide, preferably an F-box polypeptide which further comprises a WD domain. In some embodiments, the F-box polypeptide is Cdc4p, Pop1p, Pop 2p, Grr1p, Met30p, HOSp, beta TrCPp, or FWD1p. In certain embodiments, the F-box polypeptide is a polypeptide which is at least 70% identical to a contiguous polypeptide sequence of a polypeptide selected from the group consisting of SEQ ID Nos. 2, 4, 6, 8, 10, and 12. In preferred embodiments, the F-box polypeptide is encoded by a nucleic acid which is at least 80% identical to a contiguous nucleic acid sequence of SEQ ID Nos. 1, 3, 5, 7, 9, and 11. In still more preferred embodiments, the F-box polypeptide is encoded by a nucleic acid which hybridizes, preferably under conditions of high stringency to a nucleic acid selected from the group consisting of SEQ ID Nos. 1, 3, 5, 7, 9, and 11.

In other embodiments the SCF polypeptide may be any SCF polypeptide subunit, such as a mammalian or a yeast Cdc4 polypeptide, Skp1 polypeptide or cullin polypeptide. In still other embodiments, the ubiquitin protein ligase polypeptide is a HECT polypeptide, preferably an E6-AP, a Nedd-4, an RSP5, a Smurf1, a TOM1 or an EDD HECT polypeptide. In still other embodiments, the ubiquitin protein ligase polypeptide may be a yeast or a mammalian UBR1 polypeptide.

In some embodiments of the invention, the target polypeptide interaction domain is a papillomavirus E7 polypeptide, or an SV40 LTP polypeptide. In preferred embodiments,

the target polypeptide is a p107 polypeptide, an I $\kappa$ B polypeptide, a Sic1 polypeptide, a Cln2 polypeptide, an E2 polypeptide or a beta-catenin polypeptide.

In highly preferred embodiments, the invention provides a method for decreasing the level of a target polypeptide by providing an SCF recruitment domain which is operably linked to a target polypeptide interaction domain to form an SCF recruitment domain-target polypeptide interaction domain fusion protein. This SCF recruitment domain-target polypeptide interaction domain fusion protein is expressed and recruits and ubiquitinates the target polypeptide such that the level of said target polypeptide is decreased. In preferred embodiments, the SCF recruitment domain is an F-box polypeptide, preferably further comprising a WD domain, and still more preferably corresponding to a Cdc4 polypeptide, a Grr1 polypeptide, a Pop1 polypeptide, a Pop 2 polypeptide, a Met30 polypeptide, a HOS polypeptide, a betaTrCP polypeptide or a FWD1 polypeptide. In certain preferred embodiments, the F-box polypeptide is at least 70% to a polypeptide of at least 20 contiguous amino acids of a polypeptide selected from the group consisting of SEQ ID Nos. 2, 4, 6, 8, 10, and 12; or is an F-box polypeptide is encoded by a nucleic acid which hybridizes to a nucleic acid selected from the group consisting of 1, 3, 5, 7, 9, and 11.

The invention further provides a method for creating a destabilized polypeptide subject to SCF-mediated proteolysis by operably linking an SCF recruitment domain to target polypeptide. The invention further provides a method for expressing a destabilized target polypeptide subject to SCF-mediated proteolysis comprising: providing an SCF recruitment domain which is operably linked to a target polypeptide and expressing the SCF-target polypeptide fusion, thereby expressing a destabilized target protein.

In preferred embodiments, the invention provides nucleic acids for expressing an SCF recruitment domain-target polypeptide interaction domain comprising: a nucleic acid encoding an SCF recruitment domain; and a nucleic acid encoding a heterologous polypeptide domain. In preferred embodiments, the nucleic acid encoding the SCF recruitment domain and the nucleic acid encoding the heterologous polypeptide domain are operably linked so as to encode an SCF recruitment domain-heterologous polypeptide domain fusion protein. In certain embodiments of this aspect of the invention, the encoded heterologous polypeptide domain is a target polypeptide. In preferred embodiments, the encoded heterologous polypeptide domain is a target polypeptide interaction domain. In other preferred embodiments, the SCT recruitment domain is encoded by a nucleic acid which is at least 90% identical to a nucleic acid of SEQ ID Nos. 1, 3, 5, 7, 9, or 11. In more preferred embodiments, the SCT recruitment domain

is encoded by a nucleic acid which hybridizes, preferably under stringent conditions, to nucleic acid of SEQ ID Nos. 1, 3, 5, 7, 9, or 11.

In still more other embodiments, the vector comprises a nucleic acid encoding an SCF recruitment domain and a cloning site for inserting an heterologous polypeptide encoding sequence.

The invention also provides methods of treating a cell to stabilize a target polypeptide of ubiquitin protein ligase by contacting the cell with a preparation containing an effective amount of an organic compound which can competitively inhibit interaction of the target polypeptide with the ubiquitin protein ligase. In preferred embodiments, the organic compound is a peptide or peptidomimetic, preferably one which is a competitive inhibitor of a WD domain, and most preferably one which includes the general chemical structure specified by the formula:  $G-H-X^{(3-6)}-h-X-X-h-X-r-X-t^{(2-3)}-p-X-h-h-X-X-X-X-D-X-X-X-X-h-W-D$ .

Other features and advantages of the invention will be apparent from the following detailed description and claims.

### 3. Brief Description of the Figures

Figure 1 demonstrates that the F-box containing polypeptide Cdc4p is an unstable component of the SCF<sup>Cdc4p</sup> ubiquitin protein ligase complex.

Figure 2 shows that Cdc4p is ubiquitinated and proteolytically degraded by the proteasome *in vivo*.

Figure 3 demonstrates that the Cdc34p/SCF<sup>Cdc4p</sup> E3 ubiquitin ligase complex is required for proteolysis of the Cdc4p F-box protein.

Figure 4 demonstrates that functional F-box and WD polypeptide domains are necessary and sufficient for proteolysis of Cdc4p as well as an heterologous target polypeptide ( $\beta$ -galactosidase) fused to Cdc4p. Figure 4D illustrates that an F-box polypeptide-beta-galactosidase fusion protein acts as a dominant negative inhibitor of cell growth.

Figure 5 demonstrates that Grr1p, another F-box containing polypeptide, is also rapidly degraded by a ubiquitin dependent proteolysis.

Figure 6 shows the trans-targeting of a target polypeptide (X) through noncovalent interaction with a target polypeptide interaction domain (Y).

Figure 7 demonstrates the trans-targeting of tumor-suppressor retinoblastoma (pRB) by either of two retinoblastoma interaction domains: the SV40 Large T antigen



polypeptide (LTP) (Panel B); or the poapillomavirus oncoprotein E7 amino-terminus (E7N) (Panel C).

Figure 8 demonstrates the trans-targeting of pappillomavirus E2 protein.

Figure 9 demonstrates: (A) that  $\beta$ TrCP, a human homolog of Cdc4p, is a short-lived protein when expressed in a mammalian cell; and (B) that human  $\beta$ TrCP is localized to both the nucleus and cytoplasm of HeLa cells.

Figure 10 demonstrates the trans-targeting of retinoblastoma tumor suppressor (pRB) in mammalian cells and the resulting block of pRB induced growth arrest as indicated by cell morphology (panel C).

Figure 11 demonstrates the trans-targeting of endogenous human p107 in mammalian cells.

Figure 12 depicts an exemplary trans-targeting embodiment of the invention. Figure 12, panel A depicts a method for modifying the F-box protein Cdc4p by fusion to a target polypeptide interaction domain (BP) so as recruit a target polypeptide for ubiquitination and proteasome-dependent degradation. The trans-targeting of Retinoblastoma (panel B) The engineered SCF<sup>Cdc4p</sup> machinery can efficiently target the degradation of Retinoblastoma (panel B) or the papillomavirus E2 regulatory protein (panel C) in yeast.

#### **4. Detailed Description of the Invention**

##### **4.1. General**

The invention provides compositions and methods for increasing or decreasing the rate of ubiquitin-dependent proteolytic degradation of a target polypeptide in a host cell.

In preferred embodiments, the invention provides methods and reagents for targeted proteolysis through recruitment of the targeted polypeptide to a protein ubiquitin ligase. Preferred protein ubiquitin ligases include E3-type ubiquitin ligases such as the SCF ubiquitin ligases, the HECT ubiquitin ligases or the UBR1 ubiquitin ligases. The targeted polypeptide may be a natural target of ubiquitination by these ubiquitin ligases or may be a polypeptide which is not normally targeted for degradation by ubiquitin conjugation in general or by an E3-type ubiquitin protein ligase of the invention in particular. In preferred embodiments, the target polypeptide is recruited to an E3 ubiquitin ligase complex either by covalent joining of the target polypeptide to a component of the complex (cis targeting) or by noncovalent association of the target polypeptide with a component of the complex (trans targeting). The invention thereby provides for the controlled degradation of any cellular protein for which the encoding gene has

been cloned or for which an interacting polypeptide is known or can easily be elucidated by one of skill in the art. The controlled proteolysis of a target protein provides a general method for antagonizing a polypeptide activity for therapeutic, diagnostic or research purposes.

In one aspect of the invention, the invention provides for the *cis* targeting of a polypeptide. In this aspect of the invention, the targeted polypeptide may be joined to a component of an SCF (Skp1/Cull 1/F-box protein) which serves as an SCF recruitment domain. In preferred embodiments of this aspect of the invention, the SCF recruitment domain is an F-box protein and the targeted polypeptide is produced as an F-box fusion protein. The F-box polypeptide sequence may be obtained from an F-box-containing protein such as Cdc4p, Grr1p, Met30p, HOS (human homolog of Slimb), beta TrCP (Slimb), or FWD1 (mouse beta TrCP). Alternatively, an F-box containing polypeptide from a related protein may be used, or a synthetic F-box polypeptide sequence, related in structure to Cdc4p, Pop 1, Pop 2, Grr1p, Met30p, HOS, beta TrCP, Pop 1, Pop 2 or FWD1 may be used. In certain embodiments, the F-box polypeptide-target polypeptide fusion protein includes a WD-40 polypeptide region such as provided by the WD repeats of Cdc4p or as can be obtained from a large family of proteins which contain WD repeat sequences (see e.g. van der Voorn and Ploegh (1992) FEBS Lett 307: 131-4).

In preferred embodiments of the invention, the targeted polypeptide is recruited to an SCF E3 ubiquitin ligase complex by trans association with at least one component of the complex. Preferably, such trans targeting of a polypeptide or polypeptides is effected by fusing a "target polypeptide interaction domain" to an F-box polypeptide sequence. The "target polypeptide interaction domains" may be readily obtained by screening a library of naturally-occurring polypeptide sequence such as by using the yeast two-hybrid methodology (Fields and Song (1989) Nature 340:245-6; Gyuris et al. (1993) Cell 75:791-803). Alternatively, synthetic interaction domains may be obtained by any of various peptide display selection methods, such as phage display, which are known in the art.

The invention further provides methods and compositions for the inhibition of a ubiquitin ligase interaction with a target polypeptide. This aspect of the invention provides antagonists of the ubiquitin protein ligase mediated degradation of particular cellular target polypeptides. Such antagonists are useful in stabilizing ubiquitin ligase targeted cellular polypeptides, such as a tumor suppressor proteins or I $\kappa$ B, the protein inhibitor of NF- $\kappa$ B. In certain embodiments, the ubiquitin protein ligase inhibitors of the invention antagonize the interaction of an SCF ubiquitin protein ligase and a cellular target. In preferred embodiments, the inhibitors antagonize the interaction of an SCF F-box protein and a target polypeptide. For

example, cellular target polypeptides which interact with an SCF F-box protein include I $\kappa$ B, Sic1p, Cln2p, and beta-catenin. The F-box interaction /target polypeptide inhibitors of the invention are useful in stabilizing such cellular target proteins and they thereby promote a bioactivity supplied by the target polypeptide such as a cell cycle inhibitor activity, a cyclin activity, a tumor suppressor activity, a Wnt signaling activity or an NF- $\kappa$ B signaling activity. The F-box protein/target protein interaction inhibitors of the invention may be, for example, dominant negative forms of F-box polypeptides, or peptidomimetic or other small molecule inhibitors of an F-box protein/target polypeptide interaction.

In a related embodiment of the invention, methods and compositions for the identification of inhibitors of the interaction between an F-box protein and the other subunits of an SCF complex are provided. In this embodiment, inhibitors of SCF complex interaction with F-box proteins such as Cdc4p, Pop 1, Pop 2, Grr1p, Met30p, HOS, beta TrCP, Pop 1, Pop 2 or FWD1 are identified and used. These inhibitors of F-box protein/target protein and F-box protein/SCF complex interaction can be dominant negative forms of F-box polypeptides or peptidomimetic or other small molecule inhibitors of the SCF/F-box protein interaction.

In a preferred embodiment, the method comprises introducing into a host cell a chimeric polypeptide which is capable of triggering the degradation of a target polypeptide. In an even more preferred embodiment, the chimeric polypeptide recruits the target polypeptide into an E3 ubiquitin-protein ligase complex, such that the target polypeptide is ubiquitinated and degraded in the target cell. The target cell can be a eukaryotic cell, such as a mammalian cell, e.g., a human cell.

The invention is based at least on the discovery that the expression in a host cell of a "chimeric" or "hybrid" polypeptide containing: (i) a domain of a substrate binding component of an E3 ubiquitin-protein ligase complex, e.g., yeast cdc4p or the human protein h- $\beta$ TrCP, which domain provides a ubiquitination activity, e.g. by recruiting target proteins into an SCF ubiquitin protein ligase complex; and (ii) a domain capable of interacting with a pRB retinoblastoma target polypeptide, results in the degradation of the pRb target polypeptide in the host cell.

Accordingly, the invention provides methods and compositions for degrading any polypeptide of interest in a eukaryotic cell and can be used for treating any disease or conditions in which it is beneficial to degrade a particular polypeptide, e.g, a protein that provides for deregulated cellular proliferation

#### 4.2. Definitions

For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below.

The term "agonist", as used herein, is meant to refer to an agent that mimics or upregulates (e.g. potentiates or supplements) bioactivity of the protein of interest. An agonist can be a wild-type protein or derivative thereof having at least one bioactivity of the wild-type protein. An agonist can also be a compound that upregulates expression of a gene or which increases at least one bioactivity of a protein. An agonist can also be a compound which increases the interaction of a polypeptide of interest with another molecule, e.g, a target peptide or nucleic acid.

"Antagonist" as used herein is meant to refer to an agent that downregulates (e.g. suppresses or inhibits) bioactivity of the protein of interest. An antagonist can be a compound which inhibits or decreases the interaction between a protein and another molecule, e.g., a target peptide, such as interaction between ubiquitin and its substrate. An antagonist can also be a compound that downregulates expression of the gene of interest or which reduces the amount of the wild type protein present.

The term "allele", which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for that gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and/or insertions of nucleotides. An allele of a gene can also be a form of a gene containing mutations.

The term "cell death" or "necrosis", is a phenomenon when cells die as a result of being killed by a toxic material, or other extrinsically imposed loss of function of a particular essential gene function.

"Biological activity" or "bioactivity" or "activity" or "biological function", which are used interchangeably, for the purposes herein means a catalytic, effector, antigenic or molecular tagging function that is directly or indirectly performed by the polypeptides of this invention (whether in its native or denatured conformation), or by any subsequence thereof.

As used herein the term "bioactive fragment of a polypeptide" refers to a fragment of a full-length polypeptide, wherein the fragment specifically agonizes (mimics) or antagonizes

(inhibits) the activity of a wild-type polypeptide. The bioactive fragment preferably is a fragment capable of interacting with at least one other molecule, protein or DNA, with which a full length protein can bind.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The term "chimeric polypeptide" refers generally to a polypeptide comprising two subunits which do not occur together in the same polypeptide in nature, or at least, if present within the same polypeptide in nature, wherein the subunits do not occur in the same order in nature as in the chimeric polypeptide. When referring to the chimeric polypeptide of the invention, the term refers to a polypeptide comprising at least two functional subunits, a first functional subunit which recruits the chimeric polypeptide in an E3 ubiquitin-protein ligase complex, and a second functional subunit which binds to a target polypeptide.

The term "degrading" as used herein is meant to include "inducible degradation" and is used to refer to proteolytic degradation as may be facilitated by a component of the ubiquitin proteolytic pathway. Such an "inducible degradation," as referred to herein, is meant to describe the targeted degradation of a specific "target gene polypeptide."

A "delivery complex" shall mean a targeting means (e.g., a molecule that results in higher affinity binding of a gene, protein, polypeptide or peptide to a target cell surface and/or increased cellular or nuclear uptake by a target cell). Examples of targeting means include: sterols (e.g. cholesterol), lipids (e.g. a cationic lipid, virosome or liposome), viruses (e.g., adenovirus, adeno-associated virus, and retrovirus) or target cell specific binding agents (e.g., ligands recognized by target cell specific receptors). Preferred complexes are sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene, protein, polypeptide or peptide is released in a functional form.

The term "E3 complex" also referred to herein as "E3 ubiquitin protein ligase complex" and "SCF ubiquitin protein ligase complex" ("Skp1-cullin-F-box" protein complex) refers to a complex which plays a role in providing the specificity of substrate recognition for ubiquitin dependent proteolysis. Examples of E3 complexes include the SCF<sup>Cdc4p</sup> complex,

consisting of Skp1p, Cdc53p and Cdc4p, a complex which is required for ubiquitination of Sic1p. Other E3 complexes include SCF<sup>Grr1p</sup> and SCF<sup>Met30p</sup>, complexes which contain the Skp1p and Cdc53p proteins, as well as the Grr1p and Met30p, respectively.

The term "E3 substrate recognition subunit," also referred to as a "target polypeptide recognition domain" refers to the polypeptide of an E3 complex which provides E3 substrate specificity. Examples of E3 substrate recognition subunits include Cdc4p, Grr1p, Met30p and h-βTrCP. E3 substrate recognition subunits typically contain an F-box.

The term "E3 ubiquitin protein ligase recruiting domain" used interchangeably with "E3 recruiting domain" refers to a domain of an E3 substrate recognition subunit which is sufficient for interacting with the other components of an E3 complex to thereby form an E3 complex. In a preferred embodiment, an E3 recruiting domain comprises an F-box and at least one WD repeat.

The terms "epitope" and "epitope tag", as used herein, are meant to refer to any of various convenient molecular markers known in the art, such as hemagglutinin or FLAG, so that the level of a polypeptide can be confirmed in a Western blot using, for example, a suitable anti-flu or anti-FLAG antibody.

The term "equivalent" is understood to include nucleotide sequences encoding functionally equivalent polypeptides of or functionally equivalent peptides having an activity of an ---protein such as described herein. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include sequences that differ from the nucleotide sequence of the ---gene shown in SEQ ID NOs:, due to the degeneracy of the genetic code.

As used herein, the terms "gene", "recombinant gene" and "gene construct" refer to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences.

A "recombinant gene" refers to nucleic acid encoding a polypeptide and comprising ---encoding exon sequences, though it may optionally include intron sequences which are derived from, for example, a chromosomal gene or from an unrelated chromosomal gene. The term "intron" refers to a DNA sequence present in a given gene which is not translated into protein and is generally found between exons.

"Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position

in the compared sequence is occupied by the same base or amino acid, then the molecules are identical at that position. A degree of homology or similarity or identity between nucleic acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. A degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e. structurally related, at positions shared by the amino acid sequences. An "unrelated" or "non-homologous" sequence shares less than 40 % identity, though preferably less than 25 % identity, with one of the ACE-2 sequences of the present invention.

The term "interact" as used herein is meant to include detectable relationships or association (e.g. biochemical interactions) between molecules, such as interaction between protein-protein, protein-nucleic acid, nucleic acid-nucleic acid, and protein-small molecule or nucleic acid-small molecule in nature. An interaction can be direct or indirect, i.e., mediated by another molecule. Two molecules interacting directly are also referred to as binding to each other.

The term "isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding one of the subject polypeptides preferably includes no more than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks the gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

The term "modulation" as used herein refers to both upregulation (i.e., activation or stimulation (e.g., by agonizing or potentiating)) and downregulation (i.e. inhibition or suppression (e.g., by antagonizing, decreasing or inhibiting)).

The term "mutated gene" refers to an allelic form of a gene, which is capable of altering the phenotype of a subject having the mutated gene relative to a subject which does not have the mutated gene. If a subject must be homozygous for this mutation to have an altered phenotype, the mutation is said to be recessive. If one copy of the mutated gene is sufficient to alter the genotype of the subject, the mutation is said to be dominant. If a subject has one copy of the mutated gene and has a phenotype that is intermediate between that of a homozygous and that of a heterozygous subject (for that gene), the mutation is said to be co-dominant.

The "non-human animals" of the invention include mammals such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse, though transgenic amphibians, such as members of the *Xenopus* genus, and transgenic chickens can also provide important tools for understanding and identifying agents which can affect, for example, embryogenesis and tissue formation. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant gene is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that one of the recombinant genes, e.g., gene encoding a chimeric polypeptide, is present and/or expressed or disrupted in some tissues but not others.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

The term "nucleotide sequence complementary to the nucleotide sequence set forth in SEQ ID No. x" refers to the nucleotide sequence of the complementary strand of a nucleic acid strand having SEQ ID No. x. The term "complementary strand" is used herein interchangeably with the term "complement". The complement of a nucleic acid strand can be the complement of a coding strand or the complement of a non-coding strand. When referring to double stranded nucleic acids, the complement of a nucleic acid having SEQ ID No. x refers to the complementary strand of the strand having SEQ ID No. x or to any nucleic acid having the nucleotide sequence of the complementary strand of SEQ ID No. x. When referring to a single stranded nucleic acid having the nucleotide sequence SEQ ID No. x, the complement of this nucleic acid is a nucleic acid having a nucleotide sequence which is complementary to that of



SEQ ID No. x. The nucleotide sequences and complementary sequences thereof are always given in the 5' to 3' direction.

The term "percent identical" refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology, similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Expression as a percentage of homology, similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST, or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Preferably, an alignment program that permits gaps in the sequence is utilized to align the sequences. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. An alternative search strategy uses MPSRCH software, which runs on a MASPAR computer. MPSRCH uses a Smith-Waterman algorithm to score sequences on a massively parallel computer. This approach improves ability to pick up distantly related matches, and is especially tolerant of small gaps and nucleotide sequence errors. Nucleic acid-encoded amino acid sequences can be used to search both protein and DNA databases.

Databases with individual sequences are described in Methods in Enzymology, ed. Doolittle, *supra*. Databases include Genbank, EMBL, and DNA Database of Japan (DDBJ).

Preferred homologous nucleic acids have a sequence at least 70%, and more preferably 80% identical and more preferably 90% and even more preferably at least 95% identical to a nucleic acid sequence of a sequence shown in one of the sequence listings. Nucleic acids at least 90%, more preferably 95%, and most preferably at least about 98-99% identical with a nucleic sequence represented in one of the sequence listings are of course also within the scope of the invention. In preferred embodiments, the nucleic acid is mammalian. In comparing a new nucleic acid with known sequences, several alignment tools are available. Examples include PileUp, which creates a multiple sequence alignment, and is described in Feng et al., J. Mol. Evol. (1987) 25:351-360. Another method, GAP, uses the alignment method of Needleman et al., J. Mol. Biol. (1970) 48: 443-453. GAP is best suited for global alignment of sequences. A third method, BestFit, functions by inserting gaps to maximize the number of matches using the local homology.

As used herein, the term "promoter" means a DNA sequence that regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in cells. The term encompasses "tissue specific" promoters, i.e. promoters, which effect expression of the selected DNA sequence only in specific cells (e.g. cells of a specific tissue). The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. The term also encompasses non-tissue specific promoters and promoters that constitutively express or that are inducible (i.e. expression levels can be controlled).

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein when referring to a gene product. The term polypeptide includes peptidomimetics.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native polypeptide, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the polypeptide.

The term "repression" as used herein is meant to include "inducible repression" and is used to refer to transcriptional repression as by a transcriptional repressor such as a DNA

binding transcriptional repressor which binds a target promoter (a "repressible" promoter) to be repressed.

"Small molecule" as used herein, is meant to refer to a composition, which has a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention, e.g., to identify compounds that modulate the interaction between two polypeptides.

As used herein, the term "specifically hybridizes" or "specifically detects" refers to the ability of a nucleic acid molecule to hybridize to at least approximately 6, 12, 20, 30, 50, 100, 150, 200, 300, 350, 400 or 425 consecutive nucleotides of a nucleic acid.

The term "target cell" refers to a cell comprising a target polypeptide, the degradation or proteolytic stabilization of which is desired.

The term "target polypeptide" refers to a polypeptide, the degradation of which is desired in a cell.

A "target polypeptide interaction domain" refers to a polypeptide or peptidomimetic that is capable of binding to a target polypeptide.

As used herein, the term "target gene" refers to the nucleic acid which encodes a gene of interest. The target gene can be an "essential" gene, required for continued cell viability whose function is to be shut-off by the method of the invention. The term "target gene" is used to refer to any cellular gene which may be targeted for inhibition by proteolytic degradation of its encoded polypeptide. The target gene may be modified directly to effect controlled proteolysis (such as by the fusing the coding sequence to an E3/SCF-recruitment domain. The term "target polypeptide" is used interchangeably with the term "target gene polypeptide" and refers to the polypeptide gene product of the target gene as described above.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of a nucleic acid encoding a chimeric polypeptide of the invention is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., via an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., a chimeric polypeptide of the invention) which has been introduced into a cell. A transgene could be partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can also be present in a cell in the form of an episome. A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

A "transgenic animal" refers to any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a chimeric polypeptide or other polypeptide of interest. However, transgenic animals in which the recombinant chimeric gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption of one or more genes is caused by human intervention, including both recombination and antisense techniques.

The term "treating" as used herein is intended to encompass curing as well as ameliorating at least one symptom of the condition or disease.

The term "wild-type allele" refers to an allele of a gene which, when present in two copies in a subject results in a wild-type phenotype. There can be several different wild-type alleles of a specific gene, since certain nucleotide changes in a gene may not affect the phenotype of a subject having two copies of the gene with the nucleotide changes.

The term "ubiquitin" as used herein refers to an abundant 76 amino acid residue polypeptide that is found in all eukaryotic cells. The ubiquitin polypeptide is characterized by

a carboxy-terminal glycine residue that is activated by ATP to a high-energy thiol-ester intermediate in a reaction catalyzed by a ubiquitin-activating enzyme (E1). The activated ubiquitin is transferred to a substrate polypeptide via an isopeptide bond between the activated carboxy-terminus of ubiquitin and the epsilon-amino group of a lysine residue(s) in the protein substrate. This transfer requires the action of ubiquitin conjugating enzymes such as E2 and, in some instances, E3 activities. The ubiquitin modified substrate is thereby altered in biological function, and, in some instances, becomes a substrate for components of the ubiquitin-dependent proteolytic machinery which includes both ubiquitin isopeptidase enzymes as well as proteolytic proteins which are subunits of the proteasome. As used herein, the term "ubiquitin" includes within its scope all known as well as unidentified eukaryotic ubiquitin homologs of vertebrate or invertebrate origin. Examples of ubiquitin polypeptides as referred to herein include the human ubiquitin polypeptide which is encoded by the human ubiquitin encoding nucleic acid sequence (GenBank Accession Numbers: U49869, X04803) as well as all equivalents. Another example of a ubiquitin polypeptide as referred to herein is murine ubiquitin which is encoded by the murine ubiquitin encoding nucleic acid sequence (GenBank Accession Number: X51730).

The term "ubiquitin conjugation machinery" as used herein refers to a group of proteins which function in the ATP-dependent activation and transfer of ubiquitin to substrate proteins. The term thus encompasses: E1 enzymes, which transform the carboxy-terminal glycine of ubiquitin into a high energy thiol intermediate by an ATP-dependent reaction; E2 enzymes (the UBC genes), which transform the E1-S~Ubiquitin activated conjugate into an E2-S~Ubiquitin intermediate which acts as a ubiquitin donor to a substrate, another ubiquitin moiety (in a poly-ubiquitination reaction), or an E3; and the E3 enzymes (or ubiquitin ligases) which facilitate the transfer of an activated ubiquitin molecule from an E2 to a substrate molecule or to another ubiquitin moiety as part of a polyubiquitin chain. The term "ubiquitin conjugation machinery", as used herein, is further meant to include all known members of these groups as well as those members which have yet to be discovered or characterized but which are sufficiently related by homology to known ubiquitin conjugation enzymes so as to allow an individual skilled in the art to readily identify it as a member of this group. The term as used herein is meant to include novel ubiquitin activating enzymes which have yet to be discovered as well as those which function in the activation and conjugation of ubiquitin-like or ubiquitin-related polypeptides to their substrates and to poly-ubiquitin-like or poly-ubiquitin-related protein chains.

The term "ubiquitin-dependent proteolytic machinery" as used herein refers to proteolytic enzymes which function in the biochemical pathways of ubiquitin, ubiquitin-like, and ubiquitin-related proteins. Such proteolytic enzymes include the ubiquitin C-terminal hydrolases, which hydrolyze the linkage between the carboxy-terminal glycine residue of ubiquitin and various adducts; ubiquitin isopeptidases, which hydrolyze the glycine76-lysine48 linkage between cross-linked ubiquitin moieties in poly-ubiquitin conjugates; as well as other enzymes which function in the removal of ubiquitin conjugates from ubiquitinated substrates (generally termed "deubiquitinating enzymes"). The aforementioned protease activities function in the removal of ubiquitin units from a ubiquitinated substrate following or during ubiquitin-dependent degradation as well as in certain proofreading functions in which free ubiquitin polypeptides are removed from incorrectly ubiquitinated proteins. The term "ubiquitin-dependent proteolytic machinery" as used herein is also meant to encompass the proteolytic subunits of the proteasome (including human proteasome subunits C2, C3, C5, C8, and C9). The term "ubiquitin-dependent proteolytic machinery" as used herein thus encompasses two classes of proteases: the deubiquitinating enzymes and the proteasome subunits. The protease functions of the proteasome subunits are not known to occur outside the context of the assembled proteasome, however independent functioning of these polypeptides has not been excluded.

The term "ubiquitin system" as referred to herein is meant to describe all of the aforementioned components of the ubiquitin biochemical pathways including ubiquitin,, ubiquitin conjugation machinery, ubiquitin-dependent proteolytic machinery, or any of the substrates which these ubiquitin system components act upon.

The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

#### 4.3. Polypeptides and Nucleic Acids of the Present Invention

A chimeric polypeptide of the invention comprises two functional subunits. The first functional subunit comprises a polypeptide which allows the chimeric (or "hybrid") polypeptide to be targeted into the ubiquitin-dependent proteolysis pathway. This subunit is referred to generally as a "ubiquitin protein ligase polypeptide" and is also referred to herein as "E3 ubiquitin-protein ligase complex recruitment domain" or also as "E3 recruitment domain", or, more specifically as an "SCF recruitment domain" or, still more specifically, as an "F-box polypeptide" or "F-box/WD40 domain". The second functional subunit comprises a polypeptide which binds the target polypeptide and thereby recruits the target polypeptide for degradation by the ubiquitin-dependent proteolysis pathway. This second subunit is also referred to herein as the "target polypeptide interaction domain". The subunits may be derived from naturally-occurring polypeptides or may be non-naturally occurring homologs thereof.

It will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of an E3 substrate recognition subunit and/or target polypeptide binding domain for preparing a chimeric polypeptide of the invention. For example, such homologs may be useful when, e.g., a target peptide binding domain also comprises an undesirable biological activity. Thus, a chimeric polypeptide of interest derived from non-naturally occurring homologs of one or both subunits may induce degradation of a target polypeptide with fewer side effects relative to a chimeric polypeptide in which the two functional subunits derive from naturally occurring polypeptides.

Homologs of each of the subject subunit polypeptides can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the polypeptide from which it was derived. Preferred homologs for use in the invention include those which are resistant to proteolytic cleavage, as for example, due to mutations which alter enzymatic targeting of the protein. Other preferred homologs may have post-translational modifications (e.g., to alter phosphorylation pattern of protein). Modification of the structure of the subject polypeptides can be for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., *ex vivo* shelf life).

Chimeric polypeptides or subunits thereof may also be chemically modified to create derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

Modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. The substitutional variant may be a substituted conserved amino acid or a substituted non-conserved amino acid. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, *Biochemistry*, 2<sup>nd</sup> ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional homolog (e.g., functional in the sense that it has the specific biological activity required for the particular subunit, i.e., capability to form an E3 complex or capability to bind to a target polypeptide) can be readily determined by assessing the ability of the variant peptide to perform these biological activities, as further described herein. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method for generating sets of combinatorial mutants of the subject chimeric polypeptides or functional subunits thereof, as well as truncation mutants, is especially useful for identifying potential variant sequences (e.g., homologs). Combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein.

In one embodiment, the variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of sequences therein.

There are many ways by which such libraries of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene



sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential ACE-2 sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA*, Proc 3<sup>rd</sup> Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp 273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Likewise, a library of coding sequence fragments can be provided in order to generate a variegated population of polypeptide fragments for screening and subsequent selection of bioactive (i.e., having the desired biological activity, such as the capability to form an E3 complex or the capability to bind to a target polypeptide) fragments. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library of coding sequence fragments can be generated by (i) treating a double stranded PCR fragment of a coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double stranded DNA; (iii) renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products; (iv) removing single stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of homologs of chimeric polypeptides or subunits thereof. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

Combinatorial mutagenesis has a potential to generate very large libraries of

mutant proteins, e.g., in the order of  $10^{26}$  molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, PNAS USA 89:7811-7815; Yourvan et al., 1992, Parallel Problem Solving from Nature, 2., In Maenner and Manderick, eds., Elsevier Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, Protein Engineering 6(3):327-331).

The invention provides for the isolation of additional nucleic acids of the invention by means of cross-hybridization with at least one nucleic acid provided by the invention. For example, high and low stringency methodologies as described in Sanbrook et al. (1989) Molecular Cloning; Cold Spring Harbor Press) are generally useful for isolating nucleic acids which encode polypeptides for use in the instant invention, e.g. ubiquitin protein ligase encoding nucleic acids. For example, related nucleic acids can be obtained by screening a genomic or cDNA library by stringent hybridization methodology. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

The invention also provides for reduction of the chimeric polypeptides or subunits thereof to generate mimetics, e.g., peptide or non-peptide agents, such as small molecules, which are able to trigger ubiquitination and degradation of a target polypeptide. Thus, such mutagenic techniques as described above are also useful to map the determinants of the functional proteins which participate in protein-protein interactions involved in, for example, binding of the subject target binding domain to a target peptide.

#### 4.4. Ubiquitin Protein Ligase / E3 Recruitment Polypeptides

In a preferred embodiment, the first functional unit, i.e., the E3 recruiting domain", of the chimeric protein is derived from a polypeptide component of an E3 ubiquitin-protein ligase complex. In a even more preferred embodiment, the first functional subunit of the chimeric protein is from a component of the complex which interacts with the substrate to be degraded, e.g., the cdc4p, Grr1p, Met30p, h- $\beta$ TrCP subunits and homologs thereof. Accordingly, in an illustrative embodiment, the first functional subunit of a chimeric protein of the invention is a domain of a substrate recognition component of an E3 ubiquitin-protein ligase complex, which domain is sufficient for recruiting the component into the complex. This domain is also referred to herein as "E3 substrate recognition domain".

In a preferred embodiment, the first functional subunit of the chimeric polypeptide of the invention comprises an F-box or portion thereof sufficient for interaction with at least one other E3 component. In an exemplary embodiment, the first functional subunit comprises at least the F-box from the human protein h- $\beta$ TrCP (corresponding to the polypeptide of SEQ ID NO: 4 which is encoded by the nucleic acid of SEQ ID No. 3), or homolog thereof or a portion thereof. This human protein is further described in Margottin et al. (1998) *Mol. Cell* 1: 565 and can be found in GenBank (Accession No. Y14153). The F-box of this protein corresponds to amino acid 148-192 of the sequence shown in Margottin et al., supra, or SEQ ID No. 4. Another preferred first functional subunit comprises a polypeptide or homolog or portion thereof selected from the group consisting of Cdc4p, Grr1p, and Met30p. Yet other F-box containing proteins of which the F-box can be included in a chimeric polypeptide of the invention include Cyclin F; Skp2p; Pop1; C02F5.7; F48E8.7; MD6; YJL149w; N0376; 9934.4; 8039.5; N1161; SconB; Scon-2; fim; UFO; C02F5.7; C14B1.3; C17C3.6; C26E6.5; F43C9/1; F48E8.7; K10B2.1; T01E8.4; ZK328.7; Ro3D7; MD6; p110SIII; E3012.9K; and  $\beta$ TrCP (see, e.g., Margottin et al., supra; Bai et al. (1996) *Cell* 86:263; Kominami et al. (1997), *Genes Dev.* 11: 1548; Li et al. (1997) *EMBO J.* 16:5629). The amino acid sequence of WD repeats of the *S. cerevisiae* Met30p, *Neurospora crassa* Scon2p and the *Xenopus levi* proteins can be found in Margollis et al., supra. Yet other F-box proteins from which portions can be used in the invention include any of the F-box containing proteins described in Bai et al., supra, or F-box containing proteins that have not been isolated yet. Such proteins can be isolated based on the sequence homology between the F-boxes, using methods known in the art and further described herein, e.g., PCR. An alignment of the F-boxes indicates the position of conserved residues (see, e.g., Bai et al., supra)

In another preferred embodiment, the first functional subunit comprises at least one WD repeat, or at least a portion thereof sufficient for interaction with at least one other E3

component. The E3 recruiting domain may also contain at least two, at least three, at least four, at least five, at least six or any higher number of WD repeats from an E3 substrate binding component. For example, a Cdc4 polypeptide which lacks the three C-terminal WD repeats out of the seven WD repeats present in the protein, is capable of forming an E3 complex (Skowyra et al. (1997) *Cell* 91:209). The number of WD repeats that must be included in a chimeric protein of the invention, as well as which portion of the repeats must be included, can be determined as described in Skowyra or as further described herein). Preferred WD repeats for use in the invention are included in the h- $\beta$ TrCP protein having SEQ ID No. 4. As described in Margollin et al., *supra*, the first WD repeat corresponds to amino acids 260-293, the second WD repeat corresponds to amino acids 305-333, the third WD repeat corresponds to amino acids 245-373, the fourth WD repeat corresponds to amino acids 388-416; the fifth WD repeat corresponds to amino acids 428-456, the sixth WD repeat corresponds to amino acids 468-497; and the seventh WD repeat corresponds to amino acids 518-546 of the amino acid sequence of the h- $\beta$ TrCP protein. The amino acid sequence of WD repeats of the *S. cerevisiae* Met30p, *Neurospora crassa* Scon2p and the *Xenopus levi* proteins can also be found in Margollis et al., *supra*.

In an even more preferred embodiment, the first functional subunit comprises both an F-box and at least one WD repeat, or portions thereof, sufficient for recruitment into an E3 ubiquitin-protein ligase complex.

The portion of a substrate recognition component of an E3 ubiquitin-protein ligase complex that is necessary and sufficient for recruitment into the E3 ubiquitin-protein ligase complex can be determined by several methods well known in the art which do not require undue experimentations. For example, portions of E3 substrate recognition components can be contacted with at least one other component of an E3 ubiquitin-protein ligase complex and their level of interaction can be determined and compared to that of the wild-type substrate recognition component (as described, e.g., in Margottin et al.) The in vitro assay can be performed with purified, semipurified proteins, proteins expressed in a reticulocyte lysate, or expressed in a prokaryotic or eukaryotic expression system. Other assays that can be used include cellular assays, in which a portion of a substrate recognition unit, e.g., h- $\beta$ TrCP, is expressed in a cell in the form of a fusion protein by being fused to a "marker protein" (e.g., by transfection into the cell of an expression construct encoding the fusion protein). The half-life of the fusion protein in the cell is then determined. A longer half-life of the fusion protein, relative to a fusion protein comprising an essentially full-length h- $\beta$ TrCP fused to the marker protein, indicates that the

portion of h- $\beta$ TrCP is not sufficient for recruitment into an E3 ubiquitin-protein ligase complex. These and other assays are further described herein, in particular in the experimental section.

Homologs of known substrate recognition subunits can be identified using methods known in the art. For example, homologs can be cloned by hybridization at moderate or high stringency. Homologs can also be cloned by PCR using a low annealing temperature of the primers, allowing for hybridization of the primers to nucleic acids allowing for mismatches.

It is likely that substrate binding components of E3 complexes are localized in different cellular compartments. In this case, it may be advantageous for practicing the method of the invention, to use an E3 recruiting domain from an E3 substrate binding component that is naturally localized in the same cellular compartment as the target protein. Some E3 substrate recognition components are also capable of acting in several compartments. For example, h- $\beta$ TrCP is able to target both cytoplasmic (e.g., CD4, as described in the Examples), as well as nuclear (e.g., pRb, as described in the Examples) proteins for degradation by the ubiquitin proteolytic pathway. Alternatively, it is possible to modify an E3 recruiting domain or the chimeric polypeptide itself to target it to the same cellular compartment as that in which the target polypeptide is located. For example, a nuclear localization signal can be added to a chimeric polypeptide. A chimeric polypeptide can also be targeted to a specific cellular compartment by deleting certain cellular localization sequences that might be present in either or both of the E3 recruiting subunit or the target polypeptide binding subunit of the chimeric polypeptide. Cellular localization sequences are well known in the art and can be identified in a polypeptide by, e.g., using computer programs. Cellular localization sequences which can be added to a particular polypeptide to alter the cellular localization of the polypeptide are also known in the art. Cellular localization sequences are typically short polypeptides.

Alternatively, new E3 substrate recognition components can be identified, such as by assays allowing for the isolation of proteins interacting with other known components of E3 complexes. For example, two hybrid assays or phage display techniques can be used to isolate such polypeptides (these techniques are further described below).

#### 4.5. Target Polypeptide Interaction Domains

The second functional subunit of the chimeric protein of the invention is a domain allowing the recruitment of a target polypeptide to an E3 complex in a cell, to thereby degrade the target polypeptide by the ubiquitin proteolytic pathway. Accordingly, the only requirement for the second functional subunit is that it binds to the target polypeptide with sufficient affinity

that it recruits the target polypeptide into an E3 complex and thereby causes the target polypeptide to be degraded.

The target polypeptide interaction domain can be, for example, a portion of a polypeptide that is known to interact with the desired target polypeptide, or it can be a natural or synthetic polypeptide that has been identified by its ability to interact with the desired target polypeptide.

Methods for identifying polypeptides that interact with a specific target polypeptide include yeast and mammalian two hybrid assays, as well as phage display methods. These methods present the advantage that the nucleic acid encoding the interacting peptides is simultaneously identified, as opposed to other methods.

Yeast two-hybrid systems may be used to screen a mammalian (typically human) cDNA expression library, wherein a cDNA is fused to a GAL4 DNA binding domain or activator domain, and a nucleic acid encoding a polypeptide of interest, such as a target polypeptide, is fused to a GAL4 activator domain or DNA binding domain, respectively. Such a yeast two-hybrid system can screen for cDNAs encoding proteins which bind to polypeptides of interest. For example, a cDNA library can be produced from mRNA from a cell line that expresses the target polypeptide. Such a cDNA library cloned in a yeast two-hybrid expression system (Chien et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* 88: 9578 or *Cell* 72: 233) can be used to identify cDNAs which encode proteins that interact with the target polypeptide and thereby produce expression of the GAL4-dependent reporter gene.

Polypeptides which interact with the target polypeptide can also be identified by immunoprecipitation of target polypeptide with antibody and identification of co-precipitating species. Further, polypeptides that bind the target polypeptide can be identified by screening a peptide library (e.g., a bacteriophage peptide display library, a spatially defined VLSIPS peptide array, and the like) with a NF-AT<sub>c</sub> polypeptide. Accessory proteins may also be identified by cross-linking *in vivo* with bifunctional cross-linking reagents (e.g., dimethylsuberimide, glutaraldehyde, etc.) and subsequent isolation of cross-linked products that include a target polypeptide. For a general discussion of cross-linking, see Kunkel et al. (1981) *Mol. Cell. Biochem.* 34: 3, which is incorporated herein by reference. Preferably, the bifunctional cross-linking reagent will produce cross-links which may be reversed under specific conditions after isolation of the cross-linked complex so as to facilitate isolation of the target polypeptide binding protein from the target polypeptide. Isolation of cross-linked complexes that include a target polypeptide is preferably accomplished by binding an antibody that binds a target polypeptide

with an affinity of at least  $1 \times 10^7 \text{ M}^{-1}$  to a population of cross-linked complexes and recovering only those complexes that bind to the antibody with an affinity of at least  $1 \times 10^7 \text{ M}^{-1}$ . Identification of polypeptides that are cross-linked to the target polypeptide will then allow the newly identified polypeptides to be used as target polypeptide binding domains in the method of the invention.

The method of the invention includes various methods for isolating a target polypeptide interaction domain with any selected target polypeptide by using techniques which are known in the art. Such techniques include the yeast two-hybrid interaction trap and related methods, yeast cytoplasmic two-hybrid methods, mammalian two-hybrid methods, Far Western protocol methods, phage display technology-based methods, protein trap plus nucleic acid "snag" methods, surface plasmon resonance-based biomolecular interaction analysis methods, and polypeptide matrix display technologies. Preferred embodiments of the method, including yeast and mammalian two-hybrid, protein snag, Far Western and phage display technologies, have the distinct advantage of providing for both the rapid identification of a target polypeptide and the facile isolation of a corresponding a target polypeptide-encoding nucleic acid. Some of the embodiments of the ubiquitin trap method are summarized below in Table 1. As this table illustrates, each embodiment of the ubiquitin trap method employs distinct preferred forms of the TAG molecule, which is operably linked to the a target polypeptide, and the optional molecular marker which is operably attached to the a target polypeptide.

As summarized in Table I these general methods of cloning interacting proteins by virtue of their affinity for a target protein are all understood to be aspects of the immediate invention.

TABLE I

METHOD	TAG molecule- [target polypeptide]	Marker molecule- [target interaction domain]	Reference
1. Yeast Two-Hybrid or Interaction Trap	GAL 4 or lex A Polypeptide [DNA Binding Domains]	B42, VP16, or GAL4 Polypeptide [Transcriptional Activation Domains]	(Gyuris et al. (1993) Cell 75: 791-803) & (Fields et al. (1994) Trends in Gen. 10: 286-92)
2. Yeast Cytoplasmic Two-Hybrid [SRS, SOS Recruitment System]	Src Polypeptide [myristoylation signal]	hSos Polypeptide [GEF, mammalian guanyl nucleotide exchange factor]	(Aronheim et al. (1997) Mol. Cell. Biol. 17: 3094-3102)
3. Mammalian Two-Hybrid or Interaction Trap	GAL 4 or lex A Polypeptide [DNA Binding Domains]	VP16 Polypeptide [herpes simplex virus transcriptional activator]	(Luo et al. (1997) BioTechniq. 22:350-2)
4. Far-Western [related to Western except detection is by interaction with a protein other than an antibody]	Radioactive atoms, Epitope Tags, Affinity Tags [e.g. <sup>35</sup> S-met, <sup>32</sup> P, or <sup>125</sup> I; HA or FLAG; or biotin or polyHis]	None, or Expression Vector Fusion Polypeptide	(see e.g. Bonardi, et al. (1995) Bioch. Biophys. Res. Comm. 206: 260-5)
5. Phage Display	Affinity Tags [e.g. biotin, polyHIS to facilitate immobilization to a solid support matrix]	bacteriophage coat protein [e.g. filamentous phage gIII or gVIII coat protein]	(see e.g. Smith (1985) Science 228: 1315-17 & Johnson et al. (1993) Curr. Opin. Struc.Bio. 3: 564)
6. Protein Trap + Nucleic Acid Snag [Affymax Peptide Library & Screening Method]	Affinity Tags [e.g. biotin, polyHIS to facilitate immobilization to a solid support matrix]	lac repressor protein [lacI; with lac operator incorporated into the expression vector]	(U.S. Patent Nos. 5,270,170; 5,338,665; & 5,498,530)
7. Biomolecular Interaction Analysis [e.g. Pharmacia BIAcore surface plasmon resonance detection]	Affinity Tags [e.g. polyHIS to directly link to a Ni-based chip or a DNA binding domain to link to a detection chip via a DNA oligo]	None, Affinity Tag [isolated proteins may be used w/o prior modification; method of producing protein may, in some instances introduce an affinity purification tag]	(see e.g. Fivash et al. (1998) Curr. Opin. Biotechnol. 9: 97-101 & Schuck (1997) Annu. Re. Biophys. Biomol. Struct. 26: 541-66)
8. Peptide Matrix Arrays [e.g. Affymax combinatorial peptide matrix arrays]	None; or modified to support detection [e.g. fluorescently tagged]	Solid Support Matrix [in situ synthesis of random polypeptides an associated identification tag address]	(e.g. U.S. Patent Nos 5,653,939; 5,679,773; 5,690,894; 5,708,153; & 5,744,305)



The various embodiments of the various target polypeptide interaction domain isolation methods as summarized in Table I and discussed in general above, are described in detail below.

In a preferred method, a target polypeptide interaction domain is obtained using the yeast "two-hybrid" or "interaction trap" method. Specific protein-protein interactions are essential to the function of important cellular processes. The yeast two-hybrid or interaction trap assay has been developed as a means of detecting specific protein-protein interactions thereby allowing for the assessment of such interactions between known components of a biochemical pathway or macromolecular assemblage as well as allowing for the cloning of novel components of such pathways and assemblages. One aspect of the present invention pertains to the use of target polypeptide to clone other target polypeptide system proteins including proteins of the target polypeptide conjugation machinery and proteins of the target polypeptide proteolytic machinery as well as the substrates thereof. In a preferred embodiment of the invention, mammalian target polypeptide is used as the "bait" in a two hybrid or interaction trap cloning procedure. Briefly, the yeast two-hybrid assay relies upon the detection of a transcriptional activation signal delivered to a reporter gene. This transcriptional activation signal is generated by the reconstitution of a reporter gene-specific transcriptional activator from covalently separate DNA binding and transcriptional activation domains via a specific protein-protein interaction. The yeast two-hybrid assay has been applied to the analysis of a number of important cellular processes, including the proteins responsible for the regulation of the cell cycle (see e.g. U.S. Pat Nos. 5,580,736 & 5,695,941 (Brent)) and proteins involved in a macromolecular complex which controls the binding of transcriptional activators to chromatin (see e.g. the Example section of U.S. Pat. No. 5,667,973 (Fields and Song)).

Two-hybrid or Interaction trap systems are based on the finding that most eukaryotic transcription activators are modular and can be divided into two distinct domains, a DNA binding domains and a transcriptional activation domain. Furthermore, it has been shown that the DNA binding domain does not have to be covalently linked to the activation domain, so long as the two separate polypeptides interact with one another, for the complex to function as a transcriptional acitvator, (Ma et al. (1988) *Cell* 55:443-446). The interaction trap system relies upon this fact to clone proteins which interact with one another by virtue of their ability to noncovalently reconstitute a transcriptional activator when they are independently fused to the two distinct domains of a "third party" synthetic transcriptional activator. In particular, the

method makes use of two chimeric genes which independently express a DNA binding domain hybrid protein and a transcriptional activation domain hybrid protein.

To illustrate, a first hybrid gene comprises the coding sequence for a DNA-binding protein, such as the bacterial repressor protein *lexA*, fused in frame to the coding sequence for a target polypeptide polypeptide, such as the polypeptide target polypeptide itself as a particular example of the method. The second hybrid gene comprises the coding sequence for a transcriptional activation domain, such as the transcriptional activator sequence B42 (Ma and Ptashne (1987) Cell 51:113-119), fused in frame to a sample gene from a cDNA library which encodes a selection of test polypeptides representing various target polypeptide interaction domain candidates. The target polypeptide polypeptide of the first hybrid gene is commonly referred to as the "bait" while the polypeptide product of the sample gene component of the second hybrid gene is commonly referred to as the "prey". The first hybrid gene can also be thought of as expressing a specific form of the "target polypeptide trap" of the present invention as it generally conforms to the above stated definition of a target polypeptide trap (ie. target polypeptide-TAG, wherein the target polypeptide moiety is target polypeptide and the TAG entity is the *lexA* DNA binding domain). Both the first hybrid gene and the second hybrid gene are expressed simultaneously in a yeast cell. If the bait and prey hybrid proteins are able to interact, e.g., form a target polypeptide/target polypeptide-interacting protein complex, they bring into close proximity the *lexA* DNA binding domain and the B42 synthetic activation domain, thereby reconstituting a transcriptional activator protein with the DNA recognition specificity of *lexA*. A third hybrid gene contained in the same yeast cell is then used to detect the presence of this "noncovalently" reconstituted transcriptional activator. The third hybrid gene comprises a reporter gene which is operably linked to a DNA sequence comprising a binding site for the DNA-binding domain of the first hybrid gene, in this case the *lexA* operator. The "noncovalently" formed transcriptional activator (in this case *lexA*/B42) recognizes this *lexA* DNA binding sequence linked to the reporter and causes the expression of this third hybrid reporter gene which can be detected and used to score for the interaction of the *GLC1A* and sample proteins.

In a preferred embodiment of the invention, two or more reporter genes, each operably linked to the same DNA binding recognition sequence, are present in the same yeast cell in the presence of the first and second hybrid genes. As an example, one of the reporters could encode an easily assayed heterologous enzyme activity, such as the bacterial *lacZ* gene which encodes a beta-galactosidase enzyme activity capable of being detected and measured

using a chromogenic substrate such as X-gal which is converted to a blue chromophore in the presence of beta-galactosidase enzyme activity. Further, the same cell could contain a second reporter gene comprising the coding sequence for the yeast *LEU2* gene. The same haploid yeast cell would also preferably contain a deleted or otherwise mutant allele of the naturally occurring chromosomal copy of the *LEU2* gene, thereby making growth on leucine deficient media solely dependent upon expression of the *LEU2* hybrid reporter gene. If the product of a first hybrid gene, *lexA*-target polypeptide for example, interacts with the product of a second hybrid gene, the synthetic activator B42 fused to a target polypeptide conjugating enzyme (UBC or E2activity) for example, then the resulting reconstituted third party transcriptional activator *lexA*/B42, would bind to and activate both of the third hybrid gene reporters resulting in both the complementation of this yeast strain's leucine auxotrophic phenotype, due to activation of the *LEU2* reporter, and blue colony color on X-GAL containing media, due to activation of the *LacZ* reporter.

In another embodiment of this two hybrid or interaction trap screen, one of the two third hybrid gene reporters is the yeast *HIS3* gene and the haploid yeast cell also contains a deleted or otherwise mutant allele of the naturally occurring chromosomal copy of the *HIS3* gene, thereby making growth on histidine deficient media solely dependent upon expression of the *HIS3* hybrid reporter gene. In this instance, the protocol can be adapted for use either with first hybrid genes which otherwise independently (*i.e.* cryptically) weakly activate transcription on their own in the absence of the second hybrid gene product or in the specific identification and isolation of proteins that interact with the product of the first hybrid gene to such a degree that the resulting expression of the third hybrid gene reporters is of a sufficient strength so as to surpass a predetermined threshold. These applications are made possible by the addition of Aminotriazole (3-amino-1,2,4-triazole or 3-ATZ) to the media used in the screen. 3-Aminotriazole is a competitive inhibitor of the histidine anabolic enzyme activity encoded by the *Saccharomyces cerevisiae* *HIS3* gene product (see *e.g.* Erickson and Hannig (1995) *Yeast* 11: 157-67). The addition of 3-ATZ to media lacking histidine results in a condition where the abovementioned yeast strain must evince sufficiently strong interaction between the first and second hybrid gene products so as to create a sufficiently high steady state level of the reconstituted third party transcriptional activator, thereby stimulating *HIS3* reporter expression sufficiently so as to overcome the competitive inhibition of the *HIS3* gene product by 6-ATZ. As stated above, this technique is also adaptable to instances wherein the abovementioned target polypeptide-TAG first hybrid gene product is sufficient to cause activation of the third hybrid

gene reporters on its own, in the absence of a second hybrid or "prey" gene product. In this instance where the "bait" is found to cryptically activate transcription on its own, or is known to function naturally as a transcriptional activator, 3-ATZ can be added to suitable media lacking histidine until the appropriate level of 3-ATZ sufficient to block the level of activity of the product of the *HIS3* reporter expressed in the presence of the first hybrid gene alone. This level of 3-ATZ can then be added to the media on which the two hybrid screen is performed so that complementation for growth on histidine deficient media now depends upon the higher levels of expression of the *HIS 3* reporter obtained when the product of the first hybrid gene interacts with the product of the second hybrid gene as compared to the level of expression obtained from the *HIS 3* reporter in the presence of the first hybrid gene alone.

In other embodiments of this two hybrid or interaction trap version of the target polypeptide trap method, any of a number of the elements of the system can be modified. For example, Fields and his coworkers (see e.g. U.S. Pat No. 5,667,973) devised one version of the interaction trap in which the DNA binding entity of the first hybrid gene product is the DNA binding domain of the yeast transcriptional activator GAL4, but can otherwise be the DNA binding domain of any transcriptional activator having separate DNA-binding and transcriptional activation domains such as those of the yeast GCN4 and ADR1 proteins, and the transcriptional activation domain of the second hybrid gene product is the GAL4 transcriptional activation domain. In this case, a yeast strain which is null or deficient for its normal chromosomal copy of the GAL4 gene and which contains first and second hybrid gene clones encoding bait and prey products that interact, can be selected for directly on media containing galactose as the sole carbon source because the reconstituted third party transcriptional activator (GAL4 DNA BIND//GAL4 TSX ACT) will drive expression of the necessary galactose catabolic enzyme activities including the products of the *GAL1* and *GAL10* genes. If the same yeast strain also contains a *GAL1-lacZ* third hybrid gene reporter, then these same transformants can also be screened for blue color on X-gal/galactose media where the intensity of blue color detected will be directly related to the strength of the interaction between the products of the first and the second hybrid gene. In some instances, it may be preferable that the yeast contain another hybrid gene, such as *GAL1-HIS3*, in which the *GAL1* transcriptional regulatory sequences are fused to the structural gene of *HIS3*. This third hybrid gene allows for direct selection of target polypeptide-interacting target polypeptide interaction domain prey second hybrid genes by growing the yeast strain on galactose media in the absence of exogenous histidine. In this particular example, the use of 3-ATZ in the growth media, as described above, can be applied

in situations where the first hybrid gene alone serves as a weak transcriptional activator or where only very strong target polypeptide-interacting target polypeptide interaction domains are desired.

The method of the present invention allows for the use of any of a number of different reporter genes whose expression is driven by the physical association of the first and second hybrid genes. The choice of reporter gene will depend upon the particular circumstances such as the ease of selection or assay of such genes. Such genes include, without limitation, lacZ, amino acid biosynthetic genes (e.g. the yeast LEU2, HIS3, TRP1, or URA3 genes, nucleic acid biosynthetic genes, the mammalian chloramphenicol acetyltransferase (CAT) gene or GUS gene, or any surface antigen gene for which specific antibodies are available. Reporter genes may encode any enzyme that provides a phenotypic marker, for example, a protein that is necessary for cell growth or a toxic protein leading to cell death, or one encoding a protein detectable by color assay or one whose expression leads to an absence of color. Particularly preferred reporter genes are those encoding fluorescent markers, such as the GFP gene (Green Fluorescent Protein gene and variants thereof). Reporter genes may facilitate either a selection or a screen for reporter gene expression, and quantitative differences in reporter gene expression may be measured as an indication of interaction affinities.

It is understood that the method of the present invention allows for the use of any of a number of DNA binding domains in the construction of the first hybrid gene. Thus, in addition to lexA and GAL4 DNA binding domains, other DNA binding domains that are well known in the art include the DNA binding portions of the proteins ACE1, (CUP1), lambda cI, lac repressor, jun, fos, or GCN4. The method provides for the use of these alternative DNA binding domains by way of additionally altering the third hybrid or reporter gene construct (or constructs) such that it contains a fragment of DNA encompassing the binding site of the alternative DNA binding domain, and wherein said binding site is operably linked to the reporter gene(s).

The molecular marker of the test polypeptide is meant to facilitate identification of a target polypeptide interaction domain and isolation of its encoding nucleic acid. In the yeast two-hybrid embodiment of the target polypeptide trap invention, the molecular marker is typically a transcriptional activation domain which functions in yeast and which is a component of the second hybrid gene. It is understood that the second hybrid gene of the present invention can encode any of a number of alternative transcriptional activation domains including the GAL4 transcriptional activation domain region II, the strong transcriptional activator VP16, the weak

synthetic transcriptional activators B17 and B112, or the amphipathic helix domain described in Giniger and Ptashne ((1987) Nature 330:670). Modifications of the transcriptional activation can be particularly useful when attempting to either increase or decrease the sensitivity of the target polypeptide trap screen. In the method of the present invention the second hybrid gene may further contain, in addition to a transcriptional activation domain, an optional nuclear localization sequence, such as that of the SV40 Large T antigen encoded by the amino acid sequence PPKKKRKVA, which allows for the requisite partitioning of the product of the second hybrid gene in cases where the prey moiety is normally exclusively cytoplasmic. The second hybrid gene may additionally contain an epitope tag, such as hemagglutinin or FLAG, so that production of full length second hybrid gene prey products can be confirmed in a Western blot. Furthermore, as explained below, the prey epitope tag provides a convenient means of testing for covalent linkage of the bait and prey moieties as is anticipated in some applications of the method of this invention. This determination is conveniently made by means of a Western blot analysis and provides a biochemical means of classifying the clones obtained from a target polypeptide trap screen.

It is further understood that in the method of the present invention the nature of the sample gene cDNA used in constructing the second hybrid gene can be tailored to various applications of the target polypeptide trap cloning method. In particular, cDNAs may be constructed from any mRNA population and inserted into an equivalent vector for the expression of the second hybrid gene. Such a library of choice may be constructed de novo using commercially available kits (e.g., from Stratagene, LaJolla, CA) or using well established preparative methods. Alternatively, a number of cDNA libraries (from a number of different organisms) are publicly and commercially available; sources of libraries include, e.g. Clontech (Palo Alto, CA) and Stratagene (La Jolla, CA) as well as publicly available libraries such as those described and summarized on the internet (see [www.fccc.edu/research/labs/golemis/IT\\_libraries.html](http://www.fccc.edu/research/labs/golemis/IT_libraries.html)).

It is worth noting that many commercially available yeast two-hybrid systems have been created, many of which have particular advantages and all of which are understood to be adaptable to, and therefore aspects of, the present invention. For example, the Invitrogen (Carlsbad, CA) Hybrid Hunter™ System makes use of the drug Zeocin and a drug resistance marker (Zeo<sup>R</sup>) to maintain selection for the first hybrid gene encoding vector. This modification of the method allows greater compatibility with other yeast two-hybrid libraries (i.e. prey or second hybrid gene encoding vector systems) as well freeing a useful selectable prototrophy

marker for use in modifications of the standard two hybrid protocol. Such modifications of the standard two hybrid protocol may involve, for example, the introduction of a library of test polypeptides to identify proteins capable of potentiating a target polypeptide interaction with another protein it would not normally directly interact with (see e.g. the "three hybrid" system described in SenGupta et al. (1996) Proc. Natl. Acad. Sci. USA 93: 8496-8501). This modification of the system may be useful in identifying polypeptide agonists of the target polypeptide system. Alternatively, a library of test polypeptides may be introduced into a yeast strain already expressing a target polypeptide/target polypeptide interaction domain first hybrid gene/second hybrid gene interaction pair and polypeptides capable of disrupting this interaction may be selected (see e.g. the "split hybrid" system described in Shih et al. (1996) Proc. Natl. Acad. Sci. USA 93: 13896-901). This modification of the system may be useful in identifying polypeptide antagonists of the target polypeptide system. Still other methods for identifying molecular agonists and antagonists of the target polypeptide system are described in further detail in the diagnostic and therapeutic applications section. These alternative approaches to the development of therapeutic applications are herein incorporated into those sections.

It is further noted that the prey proteins do not need to be naturally occurring full-length polypeptides. For example, a prey protein may be encoded by a "domain" library of small partial cDNA sequences which can be obtained by internal priming of cDNA synthesis with random (non-polyT) primers and selection of appropriate sized partial cDNA fragments (e.g. <1kb). Alternatively the prey entity encoded by the second hybrid gene may correspond to a synthetic sequence or may be the product of a randomly generated open reading frame or a portion thereof. This particular embodiment is also usefully employed in the development of therapeutics which modulate the activity of the target polypeptide trap moiety. This particular embodiment of the target polypeptide trap method, in which a purely synthetic target polypeptide system interacting protein is sought, is also readily adapted to the phage display and peptide matrix display embodiments of the present invention.

In still other applications of the yeast two-hybrid system, the first and second hybrid genes are independently expressed in haploid yeast strains of opposite mating type. For example, a single homogeneous strain expressing a first hybrid gene (target polypeptide-TAG) "bait" can be established by transforming a yeast strain having the appropriate two-hybrid driven (e.g. GAL4<sub>OP</sub> or lexA<sub>OP</sub>-driven) third hybrid gene selectable markers and/or reporters with the first hybrid gene encoding vector. A heterogeneous population of second hybrid gene expressing yeast cells is then created by means of high efficiency transformation of a second haploid yeast

strain of opposite mating type. This heterogeneous population is then mated to the yeast strain of opposite mating carrying the first hybrid gene. Test polypeptides encoded by the second hybrid gene population which constitute target polypeptide-interacting target polypeptide interaction domain's can be selected for by requiring expression of the third hybrid gene selectable marker. This can be achieved by plating on the appropriate selective media. This "mass mating" protocol obviates repetition of the most difficult step (i.e. high-efficiency transformation of a yeast strain with a second hybrid gene encoding "prey" library) when performing repeated screen with different target polypeptide first hybrid gene "baits". Thus many different target polypeptide trap targets can be screened simultaneously or in rapid progression. This configuration thus facilitates many embodiments of the target polypeptide trap method - such as the use of a target polypeptide interaction domain, obtained in a first round of screening with the target polypeptide trap method, as a target polypeptide in a subsequent round of screening. This technique also facilitates the assembly of a "global grid" analysis of the entire target polypeptide system where the potential interaction of every target polypeptide system protein is tested against every other target polypeptide system protein revealing every point of interaction in the target polypeptide biochemical pathway network. Indeed, it is a goal of the yeast two-hybrid analysis of the target polypeptide system described here to develop a hierarchy of systematic matings assays. This "Global Grid" would provide a first-step functional characterization of each component as it is identified. Specific examples of this analysis are described in the Examples section of this application.

Since other eukaryotic cells use a mechanism similar to that of yeast for transcription, such cells, including mammalian cells such as HeLa, can be used instead of yeast to test for protein-protein interactions with the target polypeptide trap invention. In particular, the method of the present invention can be employed in a mammalian two-hybrid assay (Luo et al., (1997) *Biotechniques* 22:350-352). In this adaptation of the yeast two-hybrid system, the proteins of the first and second hybrid genes are expressed from mammalian promoters in a mammalian cell. As in the yeast nuclear two-hybrid method described above, the target polypeptide-TAG is encoded by a first hybrid gene, in which the TAG moiety is a polypeptide DNA binding domain, and a library of test polypeptides is expressed by a population of second hybrid genes comprising a collection of cDNA sequences fused to a polypeptide transcriptional activation domain. In one example of a preferred embodiment, interaction of the prey tagged with a VP16 transcriptional activation domain with a bait fused to a GAL4 DNA binding domain drives expression of reporters that direct the synthesis of Hygromycin B phosphotransferase,



Chloramphenicol acetyltransferase, or CD4 cell surface antigen (Fearon et al. (1992) *PNAS* 89:7958-62). In another, interaction of these first and second hybrid gene products drives the synthesis of SV40 T antigen, which in turn promotes the replication of the prey plasmid, which carries an SV40 origin (Vasavada et al. (1991) *PNAS* 88:10686-90). Suitable promoters for expression of the first hybrid gene bait and the second hybrid gene prey in mammalian cells include strong viral promoters such as those from CMV and SV40 (refs.?????) or weaker cellular promoters such as that from the tk (thymidine kinase) gene. The vectors that express these hybrid gene products are cotransfected with a third hybrid gene encoding a reporter such as chloramphenicol acetyltransferase (CAT) or beta-galactosidase into a mammalian cell line. The reporter of the third hybrid gene, which contains upstream DNA binding sites specific for the DNA binding domain of the first hybrid gene, can alternatively be integrated into the mammalian genome by prior transfection, selection, and clonal isolation and characterization. If the two hybrid gene products interact, there will be a significant increase in the expression of the reporter gene which can be detected and assayed using the appropriate reagents. This technique, by using small tissue culture samples, can be adapted for use in high throughput screens. The mammalian two-hybrid has two main advantages: assay results can be obtained within 48 hours of transfection and protein interactions in mammalian cells may better mimic actual in vivo interactions, particularly in the case where the relevant interaction is dependent upon mammalian post-translational modifications (including phosphorylations and glycosylations) of the first and/or second hybrid gene product or in instances where the products of the first and second hybrid gene interact indirectly through joint contact with a third molecule (such as a protein) which is endogenous to mammalian cells but not to yeast cells.

The conventional two-hybrid system, as described above, is based on a transcriptional readout and may not be suitable for either identifying transcriptional repressors or analyzing the binding partners of a transcriptional activator. A transcriptional repressor may, for example, prevent transcriptional activation resulting from recruitment of the second hybrid gene product's polypeptide transcriptional activation domain, while a target polypeptide which functions as a transcriptional activator may mask transcriptional activation resulting from productive recruitment of a second hybrid gene's target polypeptide interaction domain-polypeptide transcriptional activation domain. A novel screen for detecting protein-protein interactions that is not based directly on the formation of a hybrid transcriptional activator has been developed by Michael Karin and his colleagues and termed the SRS or SOS recruitment system (Aronheim et al. (1997) *Mol. Cell. Biol.* 17:3094-3102). As summarized in Table I, in

this embodiment of the target polypeptide trap, the polypeptide TAG is typically a Src derived polypeptide myristoylation signal, which when expressed in vivo is joined to a membrane lipid and directed to the cell membrane; while the target polypeptide interaction domain molecular marker is typically a guanyl nucleotide exchange factor such as mammalian hSos. This cytoplasmic two hybrid assay system involves the use of a defective ras/raf cytoplasmic signaling pathway in yeast. (White, et al. (1995) Cell 80:433-541). In this system, the mammalian guanyl nucleotide exchange factor (GEF) hSos is recruited to the plasma membrane in a *Saccharomyces cerevisiae* strain harboring a temperature-sensitive Ras GEF. At nonpermissive temperatures, the Cdc25-2 allele of Ras GEF is inactive and thus growth becomes dependent on the ability of a heterologous protein/protein interaction to facilitate recruitment of hSos to the plasma membrane, resulting in the stimulation of the Ras-dependent signaling cascade. The two hybrid genes necessary to utilize this system are analogous to the first and second hybrid genes of the yeast two-hybrid method described above, except a membrane localization signal, as opposed to a transcriptional activation signal, is reconstituted from these two components as detailed below. In the SRS system the first hybrid gene corresponds to a DNA encoding a myristoylation signal, such as that from Src, which is fused in frame to the coding sequence of a known protein of interest (or "bait"). In a preferred embodiment of the present invention, the bait is target polypeptide and the first hybrid gene is a Src myristoylation signal-target polypeptide fusion. The second hybrid gene of the SRS technique is comprised of the coding sequence for hSos fused in-frame to the coding sequence of a sample gene from a cDNA library (or "prey"). Because the interaction of bait and prey is assayed by reconstitution of a cytoplasmic signal transduction pathway necessary for growth and not a nuclear transcriptional activation activity, this embodiment is particularly well suited to the use of target polypeptide polypeptides which have transcriptional activator or transcriptional repressor properties which interfere with a conventional two-hybrid assay readout. This technique also has the advantage of avoiding problems occurring with second hybrid gene products which otherwise independently cause activation of third hybrid gene reporters. In particular, although the problem of "cryptic" activation by the bait is avoidable when using HIS3 as a reporter in the presence of aminotriazole as described above, there is otherwise no way of completely avoiding a nonspecific background of positive prey clones of a type which further test nonspecific for the original bait. Detection of an interaction of such a prey clone with the target polypeptide-TRAP product of the first hybrid gene using the cytoplasmic two hybrid detection method avoids "false negatives" from

this class of proteins which appears to nonspecifically activate reporter genes when allowed to localize to the nucleus.

There exist a number of techniques, known in the art, for cloning genes from conventional lambda cDNA expression libraries (such as lambda gt11) by virtue of the ability of their encoded gene product to interact with a protein or proteins of interest. These assays are essentially modifications of a traditional "Western" protocol (see e.g. Sambrook et al. Molecular Cloning: A Laboratory Manual CSH Press) except a non-antibody protein is substituted for the antibody used in the detection phase of the assay. It is understood that the method of the present invention includes such screening and cloning techniques as applied to screens employing a target polypeptide trap target probe. As summarized in Table I, such "Far-Western" embodiments of the target polypeptide trap method employ radioactive atoms, epitope tags or affinity tags to serve as target polypeptide "TAG" entities, and require no specific molecular marker moiety to mark the target polypeptide interaction domain candidate polypeptides. In many cases, however, the target polypeptide interaction domain polypeptides are from phage cloning vectors (e.g.  $\lambda$  gt11) as fusions to expression vector polypeptide-encoding sequences such as LacZ or LacZ fragments. Traditional "Far-Western" screening techniques typically employ phage lambda cDNA libraries produced from various sources of cellular mRNA, depending upon the application. These libraries are then plated at a low m.o.i. (multiplicity of infection) on a suitable bacterial host (e.g. E. coli XL-1 blue or BL21 (DE3) pLysE) so as to produce a high density of plaques. Typically about 1 million such plaques must be obtained for a fully representative sampling of cDNA species. The cDNA insert in such cloning vectors is typically under the control of a Lac I (Lac operon repressor) repressible promoter (e.g. that provided by the lac operator). Following the formation of lytic plaques (e.g. typically requiring incubation for 8 hours at 37 degrees Celcius (C)), nitrocellulose filters which have been presoaked in 10mM IPTG are overlayed on the plates. The IPTG induces expression of the cDNA species encoded by the phage lambda under the control of the lac promoter. The resulting plates are then incubated an additional 12-16 hours at 37 degrees C, and the nitrocellulose filters are removed and blocked in 5% nonfat dry milk in TTBS (Blotto) for 2-16 hours at room temperature (r.t.) with gentle shaking. The blocked filters are then exposed to the target polypeptide trap probe.

A number of methods for labeling the material of the target polypeptide trap for use in Far-Western screening techniques exist in the art. Suitable TAGS for labeling the target polypeptide trap target molecule for use in the target polypeptide trap cloning procedure include:

<sup>35</sup>S-met, <sup>32</sup>P, <sup>125</sup>I, antibody epitope tags (e.g. HA, FLAG, etc.), and biotin. Some of the alternative TAGS and methods for operably linking them to a target polypeptide are described in Section 4.3 above.

In a preferred embodiment, target polypeptide is used as the probe and the target polypeptide trap probe is synthesized by in vitro transcription and translation techniques which are well known in the art and available as kits from a number of sources (e.g. Promega Biotech, Madison, WI). Synthesis of the target polypeptide molecule from a suitable target polypeptide encoding vector in the presence of <sup>35</sup>S-met results in the synthesis of a <sup>35</sup>S labeled target polypeptide probe. The blocked filters produced as described above are then incubated in the presence of the <sup>35</sup>S labeled target polypeptide probe in fresh Blotto (typically 2ml/150mm filter) with gentle agitation overnight at room temperature or 4 degrees Celcius. The filters are then washed extensively with large volumes of TTBS several times to remove unbound target polypeptide probe and then dried and exposed to X-ray film overnight. Plaques which appear to be labeled by the target polypeptide probe by virtue of an affinity between the gene product encoded by the cDNA and the target polypeptide probe, are picked and subjected to several rounds of plaque purification which involves the use of the above described procedure at increasingly low plating densities so as to facilitate the removal of contaminating non target polypeptide reactive plaques.

In a preferred embodiment of this target polypeptide trap Far-Western protocol, lysates are prepared from these pure clones and phage from these are used to infect a fresh culture of E. coli which are then incubated in the presence of 1mM IPTG for 2 hours at 37 degrees Celcius. The cells are then lysed in SDS loading buffer, and the resulting lysate is run on a denaturing (e.g. SDS PAGE) protein gel along with dye labelled protein markers. The gel is transferred to nitrocellulose and probed with either the <sup>35</sup>S labeled target polypeptide probe or a negative control probe. The results of this type of Far-Western analysis thus reveal both the specificity of the interaction between the target polypeptide probe and the lambda cDNA encoded target polypeptide interaction domain protein (i.e. whether the interaction occurs only with the target polypeptide probe and not the negative control probe) and the molecular weight of the target polypeptide interaction domain. The latter information is useful in the classification of clones obtained in a target polypeptide trap screen, while the former information is necessary to determine the significance of the target polypeptide trap interaction.

The target polypeptide trap method of the invention provides for yet another configuration of the target polypeptide trap screen employing "phage display" technology. As

summarized in Table I, this embodiment of the target polypeptide trap method employs an affinity tag, or other means of immobilizing a target polypeptide to a solid support matrix, as a TAG to mark the target polypeptide; and a bacteriophage coat protein, such as filamentous phage coat protein gIII, which serves as a molecular marker of the population of test polypeptides representing target polypeptide interaction domain candidates. A phage-display library is a protein expression library, constructed in a bacteriophage derived vector, that expresses a collection of cloned protein sequences as fusions with a phage coat protein. This arrangement results in the expression of fusion proteins on the exterior of the phage particle. This disposition advantageously allows contact and binding between the recombinant binding protein and an immobilized ligand. In the method of the present invention phage clones expressing binding proteins are substantially enriched for target polypeptide trap specific binding activities by means of serial passage of phage populations which bind to an immobilized target polypeptide trap target (target polypeptide-TAG) ligand.

Phage display can be performed in a variety of formats; for their review see e.g., Smith ((1985) Science 228:1315-1317) and Johnson, et al. ((1993) Current Opinion in Structural Biology 3:564). The group of almost identical E. coli filamentous phages M13, fd, and fl are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrad et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461). In addition, there are many U.S. patents which describe this and closely related technologies in great detail (see e.g. U.S. Pat. #5,571,698; U.S. Pat. #5,580,717; U.S. Pat. #5,627,024).

In brief, this procedure involves the immobilization of the target protein (the target polypeptide trap in the method of the present invention) by, e.g., treating it with a biotinylating agent which is commercially available (Promega Biotech, Madison, WI). Treatment under the appropriate conditions results in the introduction of 1-6 biotin moieties per molecule of target protein. The biotinylated protein (e.g. target polypeptide-biotin) can then be bound essentially irreversibly to a solid support (e.g. a polystyrene dish) that is coated with streptavidin. After blocking non-specific protein absorption sites with bovine serum albumin, the streptavidin-coated plates are treated with the biotinylated protein (e.g. the target polypeptide trap), blocked with biotin, and rinsed. Alternative affinity tag systems suitable for immobilizing the target polypeptide to a solid support matrix are described in Section 4.3. The phage library can be

added to the plates which are now coated with the target protein bound to streptavidin via the biotin linker. Phage display clones which do not bind to the target polypeptide trap are then washed away under non-denaturing conditions. The remaining phage which are bound to the target polypeptide trap are eluted with an elution wash (e.g. pH 2.2 buffer). Following this initial round of selection, the "trapped" and eluted population of phage enriched for clones expressing a target polypeptide trap interacting species is then amplified by growth in a bacterial host. The entire procedure is then repeated with the resulting phage population, resulting in the further enrichment for recombinant phage expressing high-affinity target polypeptide trap interacting clones.

Just as in the two hybrid embodiment of the target polypeptide trap method, it is understood that any person of reasonable skill in the art will recognize that there are many alternative embodiments of the composition of the phage display population and the choice of the target population to be trapped will depend in part on the particular goals of the investigation. In particular the test polypeptide target polypeptide interaction domain candidates may be encoded by any of a number of natural or synthetic nucleic acid sequences, such as partial cDNA "domain" libraries or synthetic "randomized oligonucleotide" libraries, as discussed for the varied application of the yeast two-hybrid method (Section 4.3.1). For example, there are many examples of phage display libraries in which the short random polypeptides are displayed on the outer surface of the phage population. Smith and coworkers (Smith (1985) Science 228:1315-1317 and Parmley and Smith (1985) Gene 73:305-318) have demonstrated that small protein fragments (10-50 amino acids) can be displayed efficiently on the surface of filamentous phage by inserting short gene fragments into gene III of the fd phage. Such random sequences can also be created through the use of randomly synthesized oligonucleotides of an appropriate size in the creation of the phage coat protein fusion library. In several instances, such random peptide phage display libraries have been used successfully to identify small peptide inhibitors of a particular protein activity. For example, random peptide phage display libraries have been used to elucidate peptide inhibitors of Human T-Cell Leukemia Virus-I (HTLV-I) (see U.S. Pat. # 5,717,058). Thus, in a preferred embodiment of the present invention, a target polypeptide conjugating protein or a target polypeptide isopeptidase is immobilized on a solid matrix and subjected to biopanning with a phage display library of random peptides (peptide fusion phage). After one or, more preferably, two, or, still more preferably, three or more rounds of selection for phage expressing polypeptide sequences which bind to the target polypeptide trap polypeptide, the resulting phage collection is isolated and the sequences encoding the binding

polypeptides is determined by sequencing the particular oligonucleotide sequences fused to the DNA sequence of the bacteriophage coat protein. The polypeptides encoded by these sequences can then be tested for the ability to affect the activity of the target polypeptide trap conjugating or isopeptidase activity in a particular binding or enzymatic assay, many of which are known in the art.

A second application of the phage display embodiment of the target polypeptide trap method is in the creation of target polypeptide system proteins with altered binding properties. This technique has been applied, for example, in the isolation of growth hormone variants with altered binding properties (U.S. Pat. # 5,688,666). Thus, in a preferred embodiment of the present invention, a nucleic acid sequence encoding target polypeptide or a target polypeptide-like protein is fused to the carboxyl terminal domain of M13 gene III. The resulting vector is then mutated at one or more selected positions within the sequence encoding, in a preferred example, target polypeptide by either chemical or enzymatic techniques which are well known in the art thereby forming a family of related phage display vectors. The resulting collection of the target polypeptide mutant proteins displayed in phage display format is used as a first round of selection by contact the phagemid particles with a target molecule such as, in a preferred embodiment, a target polypeptide conjugating enzyme or a mutant thereof. The phagemid particles that bind are then separated from those that don't by washing, followed by elution of the specifically bound phagemids. The washing step can be adjusted to increase the stringency so that only the very highest affinity mutant polypeptides are selected. The specifically bound phagemids are then eluted using an appropriate elution buffer and the resulting selected phagemids are amplified and used in one or more additional rounds of selection (i.e. the above steps are repeated starting at the step where the phagemid particle mix is contacted with the target target polypeptide conjugating enzyme). The resulting target polypeptide mutants are potentially useful in designing therapeutic inhibitors of specific target polypeptide conjugating enzymes or discovering target polypeptide sequence variants with altered specificities (i.e. increased binding specificity for a target polypeptide conjugating enzyme or a variant thereof).

In another variation of the phage display method, immunoglobulin polypeptides are displayed on the outer surface of the phage capsid and antibody molecules specific to target polypeptide trap polypeptides are thereby identified. The preparation of combinatorial antibody libraries on phagemids is described by Kang et al., (1991) PNAS, U.S.A., 88:4363-4366; Barbas et al. (1991) PNAS, U.S.A. 88:7978-7982; Kang et al. (1991) PNAS, U.S.A. 88:11120-11123;

Barbas et al.(1992) PNAS, USA, 89;4457-4461; and Gram et al., PNAS, U.S.A. (1992) 89:3576-3580, the disclosures of which are hereby incorporated by reference. U.S. Pat #5,702,892 also describes phage-display of immunoglobulin heavy chain libraries. Such immunoglobulin expressing phage display libraries are commercially available (RPAS, Pharmacia Catalog number 27-9400-01, for example).

Another aspect of the invention concerns the use of the target polypeptide trap to screen peptide and cDNA vector libraries in which the candidate target polypeptide interaction domains are expressed as fusions to a DNA binding protein from a recombinant DNA expression vector wherein the vector itself contains a binding site for the DNA binding protein of the fusion library. The screening method results in the formation of a complex comprising the fusion protein (target polypeptide interaction domain candidate fused to a DNA binding domain, e.g. lacI) bound to both: an immobilized target polypeptide trap molecule (target polypeptide-TAG) through the peptide encoded by vector target polypeptide interaction domain cDNA, and to the recombinant DNA vector through the DNA binding domain of said fusion protein. Thus affinity purification of the target polypeptide interaction domain provides a means of simultaneously identifying a target polypeptide interaction domain and isolating the target polypeptide interaction domain's encoding cDNA vector. This method allows for an "in vitro" screen since the entire procedure can be conducted with an extract obtained from a lysed population of, e.g. an E. coli strain carrying a suitable library of target polypeptide interaction domain candidate test polypeptides expressed as fusions to a DNA binding protein such as the lac repressor (product of the lacI gene). Optimal results are obtained from bacterial libraries carried by bacterial strains which do not themselves produce a lac repressor protein (e.g lacI deletion strains, which are readily available). As summarized in Table I, in this particular embodiment of the target polypeptide trap method, the TAG entity used to mark the target polypeptide is typically an affinity tag which, as in the phage display embodiment, serves to facilitate immobilization of the target polypeptide to a solid support matrix such as a petri plate or a column resin; while the molecular marker, which identifies the target polypeptide interaction domain test polypeptide, is a DNA binding polypeptide with affinity for a nucleic acid sequence present in the test-polypeptide encoding vector. Immobilization of the target polypeptide interaction domain to the immobilized target polypeptide by protein/protein interaction therefore simultaneously results in the immobilization of the target polypeptide interaction domain-encoding vector via a protein/nucleic acid "snag" interaction which facilitates the in vitro identification of target polypeptide interaction domain encoding vector clones.



This methodology has been described elsewhere in greater detail (see U.S. Patent Nos. 5,270, 170, 5,338,665, and 5,498,530, incorporated herein by reference). Briefly, the method makes use of the stable binding of a DNA binding protein, such as that of the lac operon repressor, the product of the lacI gene, to its recognition sequence in the lac operator (lacO). In a preferred embodiment, a synthetic derivative of the lacI gene consisting to two lac headpieces joined by a linker (Kaptein et al. (1990) Bioch. Pharmacol. 40:89-96) is operably linked to a DNA encoding a random polypeptide or a ligand fragment comprising a portion of a target polypeptide interaction domain-encoded gene product which possesses affinity for a target polypeptide. The vector encoding this fusion protein is constructed so as to additionally contain a lac operator sequence (lacO) or a derivative lacO binding site such as that of the symmetric variant of lacO called lacO S which has an approximately ten-fold higher affinity for repressor than the wild-type sequence (Sadler et al. (1983) Proc. Natl. Acad. Sci. USA 80:6785-9; Simons et al. (1984) Proc. Natl. Acad. Sci. USA 81:1624-8). The resultant library is transformed into a host cell such that about at least 1 million transformants are created, and the resultant transformed host cells are cultured under conditions such that the vector encoded fusion protein is produced and binds to a binding site, e.g. a lacO sequence, located in a vector that encodes the fusion protein. The resultant population of cells is then lysed under conditions such that the fusion protein remains bound to the vector that encodes the fusion protein (e.g. lysis by lysozyme in a suitable buffer such as 35 mM HEPES [pH 7.5 with KOH], 0.1 mM EDTA, 100 mM Na glutamate, 5% glycerol, 0.3 mg/ml BSA, 1mM DTT and 0.1mM PMSF). The long half-life of certain DNA/DNA binding protein complexes such as that of the lac repressor bound to lacO (dissociation constant of  $10^{-13}$ M, and a half-life of about 30 minutes) is therefore preferred in the method of the present invention. The resulting cell lysate is contacted with a target polypeptide trap (target polypeptide-TAG) molecule under conditions such that the fusion protein remains bound to the vector that encodes the fusion protein. In a preferred embodiment, the target polypeptide trap molecule is immobilized to a column matrix and the cell lysate is passed through the column under conditions which preserve both the target polypeptide/target polypeptide interaction domain-lacI interaction and the target polypeptide interaction domain-lacI/lacO interaction. In an alternative embodiment allowing smaller "batch screens" for candidate target polypeptide interaction domain molecules, the target polypeptide trap molecule is immobilized to a suitable bead matrix (e.g. agarose or sepharose) and incubated with the cell lysate so as to allow the formation of bead-target polypeptide trap/target polypeptide interaction domain-lacI/lacO-vector DNA complexes which are then isolated by centrifugation.

Nonspecifically associated lacI/lacO-vector DNA complexes are removed by washing the column or beads with a suitable wash buffer (e.g. HEG buffer containing 35mM HEPES/KOH pH 7.5, 0.1 mM EDTA, 100mM Na Glutamate). The specifically bound vectors encoding the target polypeptide interaction domain-lacI fusion protein, are then eluted from the column or matrix under either native conditions (e.g. using IPTG [isopropylthiogalactoside] which is known to decrease the affinity of lacI for lacO binding sites) or denaturing conditions (e.g. using phenol or SDS - containing buffers which will interfere with the target polypeptide/target polypeptide interaction domain protein-protein interaction). The nucleic acid of the eluted target polypeptide interaction domain fusion protein encoding vector is then recovered using standard techniques of extraction and precipitation which are well known in the art. If desired, the recovered target polypeptide interaction domain-encoding vector DNA can be put through an additional round(s) of selection by retransforming this eluted vector nucleic acid into a suitable host cell (e.g. the host cell strain used in the previous "round" of screening) and then repeating the steps recited above. Repeated application of this "molecular snag" technique can be expected to produce vectors which encode target polypeptide interaction domain's with increasingly strong affinities for the target polypeptide polypeptide of the target polypeptide trap.

Those skilled in the art will appreciate that there are a wide variety of other DNA binding proteins, including polypeptides derived from naturally occurring DNA binding proteins, as well as polypeptides derived from proteins artificially engineered to interact with specific DNA, which can be used in the immediate application of the present invention.

Another method for studying biomolecular interactions which can be readily adapted for use in the subject invention is real-time Biomolecular Interaction Analysis (BIA). As summarized in Table I, in this embodiment of the target polypeptide trap method, the TAG entity used to mark the target polypeptide is typically an affinity tag which serves to immobilize the target polypeptide to a specialized detector surface. In this embodiment, there is no need for a molecular marker to identify the target polypeptide interaction domain, since a productive target polypeptide/target polypeptide interaction domain interaction can be directly detected through altered properties of the surface to which the target polypeptide is immobilized. In some embodiments of this method, an affinity tag molecular marker may be associated with the target polypeptide interaction domain, and may have facilitated purification of the target polypeptide interaction domain to obtain the homogeneous population of test polypeptides which is preferred in the BIA embodiment of the target polypeptide trap method.

This technique allows biospecific interactions to be studied in real time without labeling any of the interactants. Detection depends on changes in the mass concentration of macromolecules at the biospecific interface. BIA uses the optical phenomenon surface plasmon resonance to monitor biomolecular interactions. Surface plasmon resonance is an optical phenomenon arising in thin metal films under conditions of total internal reflection which relies on changes in the intensity of reflected light which occur at the resonance angle. By keeping other factors constant, surface plasmon resonance provides a measure of changes in the concentration of molecules in a surface layer of solution in contact with the sensor surface. Briefly, the system is composed of three essential elements: a sensor chip, an optical detection system, and an integrated micro-fluidic cartridge (IFC) for controlled transport of samples to the sensor surface. The sensor chip is modified to the application so that one interactant is immobilized on the sensor surface (the target polypeptide), which forms one wall of a micro-flow cell. In some applications, the ligand is attached indirectly to the surface through binding to another immobilized molecule (a technique referred to as capturing). Solution containing the other interactant(s) is streamed continuously over the sensor surface by the IFC. As molecules from solution bind to the immobilized interactant, the resonance angle changes and a response is registered. Following association of the subject free interactant molecule analyte test polypeptide target polypeptide interaction domain candidate, the target polypeptide/target polypeptide interaction domain complex dissociates at a rate proportional to the dissociation constant for this reaction. Dissociation of the target polypeptide/target polypeptide interaction domain complex can be monitored in real time by monitoring the signal obtained from the sensor surface resonance angle detector. The signal level will eventually return to that measured prior to introduction of the target polypeptide interaction domain moiety when all of the target polypeptide/target polypeptide interaction domain complex has decomposed and the target polypeptide interaction domain has diffused away. Detailed descriptions of surface resonance-based detection methods for biomolecular analysis applications may be obtained from commercial suppliers of these systems such as Pharmacia (Piscataway, NJ) which produces the Pharmacia Biosensor AB system.

The basic advantages of this embodiment of the target polypeptide trap method include real-time and label-free monitoring of target polypeptide/target polypeptide interaction domain interactions. Furthermore, reusable sensor chip technology allows multiple target polypeptide target polypeptide trap targets to be analyzed; and flexible experimental design, rapid analysis, exact sample handling technology and integrated systems analysis facilitate high

throughput screening for target polypeptide interaction domain polypeptides. These features also facilitate screening for small molecules which enhance or inhibit particular target polypeptide/target polypeptide interaction domain interactions. This method is thus well suited to screens for therapeutic target polypeptide system agonist and antagonist compounds.

The utility of the BIAcore biomolecular analysis method has been demonstrated in a number of studies (summarized in, e.g., Fivash et al. (1998) *Curr. Opin. Biotechnol.* 9: 97-101; Salamon et al. (1997) *Biochim. Biophys. Acta.* 1331: 131-52 & 117-29; Schuck (1997) *Curr. Opin. Biotechnol.* 8: 498-502; Schuck (1997) *Annu. Rev. Biophys. Biomol. Struct.* 26: 541-66; Van Regenmortel et al. (1997) *Immunol. Invest.* 26: 67-82; O'Shannessy and Winzor (1996) *Anal. Biochem.* 236: 275-83; and Szabo et al. (1995) *Curr. Opin. Struct. Biol.* 5: 699-705). These studies further provide the skilled artisan with additional information useful in guiding the application of this technique to the target polypeptide trap method described by the present invention.

In yet another embodiment, the present invention allows for the instantaneous screening of large collections of polypeptides that are synthesized on, and remain covalently bound to, the surface of a support matrix. As summarized in Table I, in this embodiment of the target polypeptide trap method the TAG moiety may be dispensable in certain detection schemes or it may be a fluorescent label or another energy transfer system which facilitates identification of a target polypeptide interaction domain-immobilized target polypeptide and its assignment to a specific matrix "address" which serves to reveal the identity of the interacting target polypeptide interaction domain. The molecular marker in this embodiment is typically a solid support matrix on which the target polypeptide interaction domain polypeptide has been synthesized and which facilitates the creation of the target polypeptide interaction domain identifying "address". Methods of producing such "protein chips" using photosensitive substrates and a precisely delivered source of light have been described (see e.g. U.S. Patent No. 5,679,773, 5,744,305 and WO 90/15070). To screen for biological activity, the substrate protein chip, representing a collection of potential target polypeptide system interacting polypeptides (target polypeptide interaction domains), is exposed to one or more target polypeptide trap polypeptides. The target polypeptide trap polypeptide is tagged, preferably with a fluorescent marker, a radioactive marker, or an antibody epitope. In still other embodiments the detection system employed obviates the need to TAG the target polypeptide employed. Examples include a electrical detection based systems (U.S. Patent No. 5,653,939 and 5,670,322), as well as an optical fiber-based detection system (U.S. Patent No. 5,690,894). The location of the marker on

the substrate is detected with, for example, photon detection or through the altered optical or electrical properties resulting from interaction of the immobilized target polypeptide interaction domain with the target polypeptide at a specific "address". Through knowledge of the sequence of the material at the location where binding is detected, it is possible to quickly determine which target polypeptide interaction domain sequence binds with the target polypeptide probe and, therefore, the technique can be used to screen very large numbers of polypeptides for target polypeptide interaction domain activity. For example, in a preferred embodiment of this protein chip technology, a fluorescently labeled target polypeptide trap polypeptide is exposed to a peptide chip matrix substrate. Nonspecifically bound target polypeptide trap polypeptides are removed and the peptide chip matrix substrate is placed in a microscope detection apparatus for identification of locations where binding takes place. The microscope detection apparatus includes, in a preferred embodiment, a monochromatic or polychromatic light source for directing light at the substrate, and means for detecting fluoresced light from the substrate, as well as means for determining a location of the fluoresced light. The means for detecting light fluoresced on the substrate may in some embodiments include a photon counter. The means for determining a location of the fluoresced light may include an x/y translation table for the substrate. Translation of the slide and data collection are recorded and managed by an appropriately programmed digital computer. Analysis of the collected data allows immediate identification of polypeptide sequences representing target polypeptide interaction domains that interact with the specified target polypeptide trap polypeptide.

The resulting identified target polypeptide interaction domain polypeptide sequences are of utility in several aspects. These synthetic sequences can be reasonably assumed, in some instances, to mimic the structures of naturally-occurring target polypeptide interaction domain ligand interaction domains. Comparison of the thus identified synthetic target polypeptide interaction domain polypeptide sequences with the existing protein databases (e.g. GenBank and SWISS-PROT.) as well as EST databases (Boguski, M. (1995) TIBS 20:295-6) therefore allows the identification of naturally-occurring candidate target polypeptide interaction domains which can then be easily obtained for further analysis. In addition, we note here that the synthetically derived target polypeptide interaction domain polypeptides identified by analysis of peptide display matrices represent potentially therapeutic compounds for use in the prevention and treatment of diseases and conditions involving components of the biochemical pathways of the target polypeptide system. In this and other applications of the target polypeptide trap method the identification of a synthetic target polypeptide interaction domain

polypeptide sequence has immediated utility independent of its application to the discovery of additional naturally-occurring target polypeptide interaction domain polypeptides.

Indeed, as it applies to the identification of compounds of utility in the alteration of target polypeptide system processes, the present invention is not limited in scope to the identification of polypeptide interactors. We note here that methods for the synthesis of diverse collections of tagged compounds attached to solid supports have been described (U.S. Patent No. 5,708,153). This technology is not limited to polypeptides but rather allows the synthesis of diverse collections of tagged compounds of random oligomeric structure. Furthermore, in preferred embodiments, these oligomeric compounds carry specific identifying tags (e.g. oligonucleotide tags) which allow amplification of the tags and identification of the monomer therewith associated. It is understood that the present invention makes available the application of this technology to the method of the target polypeptide trap, thereby allowing the identification of compounds of chemically diverse structure for use in the prevention and treatment of diseases and disorders involving the target polypeptide system.

#### 4.6. Ubiquitin Protein Ligase Inhibitors

The invention demonstrates that recruitment to a ubiquitin protein ligase is sufficient to target a polypeptide for ubiquitin conjugation and subsequent ubiquitin dependent proteolytic degradation. Accordingly, the invention also makes available methods and reagents to block or antagonize the recruitment of certain cellular proteins to ubiquitin protein ligases *in vivo*. The invention thereby provides means by which a target of a ubiquitin protein ligase can be stabilized *in vivo* by preventing recruitment to, and subsequent ubiquitination by, the ubiquitin protein ligase. In preferred embodiments, the inhibitors of the invention prevent the interaction of a ubiquitin ligase with a target polypeptide and the inhibitor is a small molecule, polypeptide, or peptidomimetic; the mechanism of inhibition may be competitive or noncompetitive; and the target polypeptide may be a cellular polypeptide, a pathogen-encoded polypeptide or a target polypeptide employed in trans-targeting embodiments of the invention.

For example, effective competitive inhibitors of the invention may be designed based upon the polypeptide structures which comprise the interaction complex between the ubiquitin protein ligase and the target polypeptide. A minimal target polypeptide/ ubiquitin protein ligase interaction complex may be characterized by deletion analysis employing protein-protein interaction assays known in the art and described in detail above for the elucidation of target polypeptide interaction domains. Additional methodologies, such as alanine-scanning

mutagenesis provide further refined information regarding the structure of the protein/protein interaction domain interface. In certain situations, the crystal structure of one or both of the binding partners may be available to provide still further guidance to the skilled artisan in designing the competitive inhibitor.

Exemplary noncompetitive inhibitors of the invention include, for example, compounds which bind to a region of the target polypeptide other than the ubiquitin protein ligase interaction region, and allosterically alter the structure of the ubiquitin protein ligase interaction region. Such noncompetitive allosteric target polypeptide inhibitors may have certain advantages - for example they may provide a high degree of specificity to the inhibition as they will fail to recognize and bind to other target polypeptides of the ubiquitin protein ligase. In certain instances, preferred noncompetitive inhibitors of the invention may be elucidated based upon known or readily determined binding partners of the target polypeptide. For example, the structure of a substrate or substrate analog of a target polypeptide enzyme may be used to design appropriate enzyme active site binding noncompetitive inhibitors. Exemplary enzyme active site binding noncompetitive inhibitors include substrate transition state analogs which bind tightly to a target enzyme active site with a high degree of specificity and allosterically alter the structure of the ubiquitin protein ligase interaction region of the target enzyme polypeptide. In another example, the structure of a target polypeptide binding partner protein is used to design the noncompetitive inhibitor of the invention. In this example, the binding partner protein interacts with a region of the target polypeptide which is different from the region recognized by the ubiquitin protein ligase. For example, the structure of the NF $\kappa$ B binding partner of I $\kappa$ B can be used to design preferred noncompetitive inhibitors of the interaction of I $\kappa$ B with a  $\beta$ TrCP containing SCF ubiquitin protein ligase.

The ubiquitin protein ligase competitive inhibitors of the invention can be designed to mimic the polypeptide structure of either the ubiquitin protein ligase component of the interaction region or the target polypeptide component of the interaction region. Such competitive inhibitors thereby prevent the recruitment of the target polypeptide to the ubiquitin protein ligase by blocking the recruitment function of the ubiquitin protein ligase. Depending upon the structure of the inhibitor, the inhibition of recruitment may be very specific to the target polypeptide, or may be affect the recruitment of other polypeptides which are targeted by the ubiquitin protein ligase. For example, preferred target polypeptide-specific inhibitors mimic the structure of a WD repeat polypeptide sequence which interacts with a target polypeptide. These "WD repeat" competitive inhibitors are designed to bind to the target polypeptide and prevent

its interaction with a WD repeat of a WD repeat containing F-box polypeptide of an SCF ubiquitin ligase. The invention thereby provides a class of competitive inhibitors which antagonize the ubiquitination of the target polypeptide and only those other targets which share a similar WD repeat interaction pocket.

In certain embodiments, the inhibitor has a molecular weight of less than 10,000 atomic mass units (amu), more preferably less than 7500 amu, 5000 amu, and even more preferably less than 3000 amu. For instance, the ubiquitin ligase/target polypeptide inhibitor can be either a peptide or peptidomimetic, preferably corresponding in length to a 3-25 mer, e.g., and in certain preferred embodiments, containing a core sequence corresponding to a WD repeat conserved sequence of G-H-X<sup>(3-6)</sup>-h-X-X-h-X-r-X-t<sup>(2-3)</sup>-p-X-h-h-X-X-X-D-X-X-X-X-h-W-D; wherein "X" indicates any amino acid residue, the number ranges indicated in superscript indicate a variable number of the indicated residue type at that position, "h" indicates a hydrophobic residue, "r" indicates an aromatic amino acid residue, "t" indicates an amino acid residue which stabilizes a tight polypeptide backbone turn such as glycine, proline, aspartic acid or asparagine, and p indicates a polar amino acid residue. In other embodiments the WD repeat competitive inhibitor is provided as a gene construct for expressing the WD repeat peptide. The WD repeat peptide, peptidomimetic or gene construct is formulated in the pharmaceutical preparation for delivery to an animal to be treated.

The invention further provides certain broad-specificity ubiquitin protein ligase inhibitors. For example, the production of a beta-galactosidase fusion to a mutant Cdc4p F-box protein lacking the WD repeat-containing domain resulted in an inhibition of cell growth in yeast (see Figure 4, panel D). This general inhibition of growth was accompanied by the stabilization of the beta-galactosidase-F-box fusion polypeptide which was otherwise targeted for cis-mediated ubiquitination and proteolytic degradation when expressed as a fusion to intact Cdc4p containing the WD repeat domain (see Figure 4, panel C). Accordingly, the invention provides a beta-galactosidase-F-box fusion protein and other inhibitors which antagonize one or more ubiquitin protein ligase activities. Such broad-range inhibitors are particularly useful for the purpose of blocking ubiquitin-conjugation and proteolytic degradation of all cellular protein targets of a particular class of SCF ubiquitin ligase - e.g. an SCF<sup>Cdc4p</sup>, an SCF<sup>Grr1p</sup>, an SCF<sup>Met30p</sup>, or an SCF<sup>hbTrCp</sup>.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such



techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

It is understood that the inhibitors of the invention can be competitive or noncompetitive, and can be general to a class of ubiquitin protein ligase or specific to a particular cellular target protein or somewhat broader in specificity to include multiple cellular protein targets. In preferred embodiments, the present invention provides a peptide, or peptidomimetic that inhibits the ubiquitin-dependent degradation of the target polypeptide. The peptide/peptidomimetic can, in certain preferred embodiments, range in size from 3-25 amino acid residues. In certain embodiments, a WD repeat inhibitor of the present invention includes a WD repeat core structure having the formula:  $G-H-X^{(3-6)}-h-X-X-h-X-r-X-t^{(2-3)}-p-X-h-h-X-X-X-X-D-X-X-X-X-h-W-D$ , wherein:

G represents a glycine residue, or an analog thereof;

H represents a histidine residue, or an analog thereof;

D represents an aspartic acid residue, or an analog thereof;

W represents a tryptophan residue, or an analog thereof;

and "X" indicates any amino acid residue, "h" indicates a hydrophobic residue, "r" indicates an aromatic amino acid residue, "t" indicates an amino acid residue which stabilizes a tight polypeptide backbone turn such as glycine, proline, aspartic acid or asparagine, and p indicates a polar amino acid residue. While the invention includes all of the groups of inhibitors set forth above, the following descriptions are illustrative of an exemplary WD repeat ubiquitin protein ligase competitive inhibitor of the invention. It is understood that chemical design and other

methods described for the WD repeat inhibitor apply broadly to all classes of inhibitor discussed herein.

The following definitions and explanations are provided in support of the description of the exemplary WD repeat and other ubiquitin protein ligase inhibitors of the invention.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C1-C30 for straight chain, C3-C30 for branched chain), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF<sub>3</sub>, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, -CF<sub>3</sub>, -CN, and the like.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

The term "aryl" as used herein includes 5-, 6- and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro; sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, -CF<sub>3</sub>, -CN, or the like. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The

heterocyclic ring can be substituted at one or more positions with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF<sub>3</sub>, -CN, or the like.

The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF<sub>3</sub>, -CN, or the like.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

As used herein, the term "nitro" means -NO<sub>2</sub>; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means -SO<sub>2</sub>-.

As used herein, the definition of each expression, e.g. alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic

compounds described herein which satisfy the valences of the heteroatoms. This invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

The phrase "protecting group" as used herein means temporary substituents which protect a potentially reactive functional group from undesired chemical transformations. Examples of such protecting groups include esters of carboxylic acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991).

The term "amino acid residue" is known in the art. In general the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (see *Biochemistry* (1972) 11:1726-1732). In certain embodiments, the amino acids used in the application of this invention are those naturally occurring amino acids found in proteins, or the naturally occurring anabolic or catabolic products of such amino acids which contain amino and carboxyl groups. Particularly suitable amino acid side chains include side chains selected from those of the following amino acids: glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan.

The term "amino acid residue" further includes analogs, derivatives and congeners of any specific amino acid referred to herein, as well as C-terminal or N-terminal protected amino acid derivatives (e.g. modified with an N-terminal or C-terminal protecting group). For example, the present invention contemplates the use of amino acid analogs wherein a side chain is lengthened or shortened while still providing a carboxyl, amino or other reactive precursor functional group for cyclization, as well as amino acid analogs having variant side chains with appropriate functional groups). For instance, the subject compound can include an amino acid analog such as, for example, cyanoalanine, canavanine, djenkolic acid, norleucine, 3-phosphoserine, homoserine, dihydroxy-phenylalanine, 5-hydroxytryptophan, 1-methylhistidine, 3-methylhistidine, diaminopimelic acid, ornithine, or diaminobutyric acid. Other naturally occurring amino acid metabolites or precursors having side chains which are suitable herein will be recognized by those skilled in the art and are included in the scope of the present invention.

Also included are the (D) and (L) stereoisomers of such amino acids when the structure of the amino acid admits of stereoisomeric forms. The configuration of the amino acids

and amino acid residues herein are designated by the appropriate symbols (D), (L) or (DL), furthermore when the configuration is not designated the amino acid or residue can have the configuration (D), (L) or (DL). It will be noted that the structure of some of the compounds of this invention includes asymmetric carbon atoms. It is to be understood accordingly that the isomers arising from such asymmetry are included within the scope of this invention. Such isomers can be obtained in substantially pure form by classical separation techniques and by sterically controlled synthesis. For the purposes of this application, unless expressly noted to the contrary, a named amino acid shall be construed to include both the (D) or (L) stereoisomers. D- and L- $\alpha$ -Amino acids are represented by the following Fischer projections and wedge-and-dash drawings. In the majority of cases, D- and L-amino acids have R- and S-absolute configurations, respectively.

A "reversed" or "retro" peptide sequence as disclosed herein refers to that part of an overall sequence of covalently-bonded amino acid residues (or analogs or mimetics thereof) wherein the normal carboxyl-to amino direction of peptide bond formation in the amino acid backbone has been reversed such that, reading in the conventional left-to-right direction, the amino portion of the peptide bond precedes (rather than follows) the carbonyl portion. See, generally, Goodman, M. and Chorev, M. *Accounts of Chem. Res.* 1979, 12, 423.

The reversed orientation peptides described herein include (a) those wherein one or more amino-terminal residues are converted to a reversed ("rev") orientation (thus yielding a second "carboxyl terminus" at the left-most portion of the molecule), and (b) those wherein one or more carboxyl-terminal residues are converted to a reversed ("rev") orientation (yielding a second "amino terminus" at the right-most portion of the molecule). A peptide (amide) bond cannot be formed at the interface between a normal orientation residue and a reverse orientation residue.

Therefore, certain reversed peptide compounds of the invention can be formed by utilizing an appropriate amino acid mimetic moiety to link the two adjacent portions of the sequences depicted above utilizing a reversed peptide (reversed amide) bond. In case (a) above, a central residue of a diketo compound may conveniently be utilized to link structures with two amide bonds to achieve a peptidomimetic structure. In case (b) above, a central residue of a diamino compound will likewise be useful to link structures with two amide bonds to form a peptidomimetic structure.

The reversed direction of bonding in such compounds will generally, in addition, require inversion of the enantiomeric configuration of the reversed amino acid residues in order

to maintain a spatial orientation of side chains that is similar to that of the non-reversed peptide. The configuration of amino acids in the reversed portion of the peptides is preferably (D), and the configuration of the non-reversed portion is preferably (L). Opposite or mixed configurations are acceptable when appropriate to optimize a binding activity.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including cis- and trans-isomers, R- and S-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts are formed with an appropriate optically-active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

Contemplated equivalents of the compounds described above include compounds which otherwise correspond thereto, and which have the same general properties thereof (e.g. the ability to bind to opioid receptors), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound in binding to opioid receptors. In general, the compounds of the present invention may be prepared by the methods illustrated in the general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures.

For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked.

Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject peptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

The term "gene construct" refers to a vector, plasmid, viral genome or the like which includes a coding sequence, can transfect cells, preferably mammalian cells, and can cause expression of e.g., the ubiquitin protein ligase-target polypeptide interaction domain hybrid of the cells transfected with the construct. The term "gene construct" does not include a wild-type papillomavirus genome, and preferably does not include expressible coding sequences for one or more of the polypeptides of the invention.

As used herein, the term "pharmaceutically acceptable" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not excessively toxic to the hosts of the concentrations of which it is administered. The administration(s) may take place by any suitable technique, including subcutaneous and parenteral administration, preferably parenteral. Examples of parenteral administration include intravenous, intraarterial, intramuscular, and intraperitoneal, with intravenous being preferred.

A variety of drug screening techniques can be readily adapted to the ubiquitin protein ligase/ target polypeptide interaction in order to provide high throughput screening of peptide, peptidomimetic or other small molecule libraries. Such assays can be used to optimize a lead compound, or to assess the potential inhibitory effect of a test compound.

In one embodiment, simple competition assays can be used to assess the ability of a test compound to disrupt the interaction of the ubiquitin protein ligase/ target polypeptide complex. In other embodiments, cell-based assays which detect a target polypeptide activity or a ubiquitin protein ligase activity can be used to assess the biological activity of a test compound.



In certain embodiments of the present invention, such as for topical administration to the epidermis, the subject inhibitor pharmaceutical can be a peptide, e.g., having a naturally occurring peptide backbone and amino acid side chains, though it may be N-terminally and/or C-terminally protected.

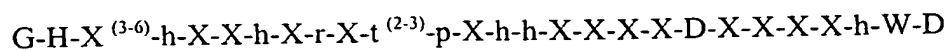
In preferred embodiments, the peptidyl component of the subject compounds includes, in addition to the core WD repeat sequences, as described herein, no more than about 25 amino acid residues of a protein in which a WD repeat motif naturally exists, more preferably no more than 10-15, and even more preferably 6 or less. With the exception of certain chimeric WD repeat compositions described herein, such as fusion proteins, a preferred composition (especially for ectopic application) includes a peptide comprising a WD repeat core motif and having a molecular weight in the range of about 1500 to 7500 daltons, more preferably from about 2000 to 5000 daltons, and even more preferably in the range of about 2000 to 2750 daltons. The peptide, in addition to the WD repeat core motif, may include other amino acid residues, such as a transcytosis peptide, and may be derivatized at one or more backbone or sidechain points with, e.g. peptides, nucleic acids, carbohydrates, etc. In certain embodiments, the peptide is derivatized with one or more functional groups that enhance cellular uptake and/or alter the half-life of the WD repeat core motif.

This invention further contemplates a method of generating sets of combinatorial libraires of the subject WD repeat peptides which is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in inhibiting WD repeat mediated interactions with target polypeptides.

Combinatorially-derived homologs can be generated which have, e.g., greater affinity, a enhanced potency relative to native peptide sequences, or intracellular half-lives different than the corresponding wild-type polypeptide. For example, the altered peptide can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of, the peptide. Such homologs can be utilized to alter the envelope of therapeutic application by modulating the half-life of the peptide. For instance, a short half-life can give rise to more transient biological effects and can allow tighter control of peptide levels within the cell.

In a representative embodiment of this method, the amino acid sequences for a population of WD motifs are aligned, preferably to promote the highest homology possible. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. To illustrate, multiple WD repeat containing proteins

are aligned and, based on these alignments, combinatorial libraries can be generated representing WD repeat peptides which have an amino acid sequence that includes a WD core sequence represented by the formula:



Peptides larger than the 15-mer core are, of course, also contemplated. Further expansion of the combinatorial library can be made, for example, by including amino acids which would represent conservative mutations at one or more of the degenerate positions. Inclusion of such conservative mutations can give rise to a library of potential WD peptide sequences represented by the above formula, but wherein Xaa(1) can be an amino acid residue having a polar sidechain, such as arg, asn, asp, cys, glu, gln, his, lys, ser, thr or tyr, as set out by the core structures above. Alternatively, amino acid replacement at degenerate positions can be based on steric criteria, e.g. isosteric replacement, without regard for polarity or charge of amino acid sidechains. Similarly, completely random mutagenesis of one or more of the variant positions (Xaa) can be carried out, e.g., each of Xaa(1)-(11) can be any of the 20 amino acids (or other analogs thereof).

In one embodiment the WD peptide library can be derived by combinatorial chemistry, such as by techniques which are available in the art for generating combinatorial libraries of small organic/peptide libraries. See, for example, Blondelle et al. (1995) Trends Anal. Chem. 14:83; the Affymax U.S. Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; Chen et al. (1994) JACS 116:2661; Kerr et al. (1993) JACS 115:252; PCT publications WO92/10092, WO93/09668 and WO91/07087; and the Lerner et al. PCT publication WO93/20242).

In a preferred embodiment, the combinatorial peptide library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential WD sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential WD nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of WD peptide sequences therein.

There are many ways by which the gene library of potential WD homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential WD

sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of WD sequences. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Such illustrative assays are amenable to high throughput analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In an illustrative embodiment of a screening assay, the WD gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical E.coli filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

For example, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening E2 motif combinatorial libraries of the present invention. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The E2 combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate E2 gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate E2 peptide, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate proteins which are capable of, for example, binding an E1 protein, are selected or enriched by panning. For instance, the phage library can be panned on glutathione immobilized E1-GST fusion proteins, and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for E2 homologs which can then be screened for further PV inhibitory activities. Subsequent selection, e.g. of a reduced set of variants from the library, may then be based upon more meaningful criteria rather than simple E1-binding ability. For instance, intracellular half-life or selectivity can become selection criteria in secondary screens.

Combined with certain formulations, such peptides can be effective intracellular agents for, e.g., inhibiting PV infection of epithelial cells. However, in order to increase the efficacy of such peptides, the WD peptide can be provided a fusion peptide along with a second peptide which promotes "transcytosis", e.g., uptake of the peptide by epithelial cells. To illustrate, the WD peptide of the present invention can be provided as part of a fusion polypeptide with all or a fragment of the N-terminal domain of the HIV protein Tat, e.g., residues 1-72 of Tat or a smaller fragment thereof which can promote transcytosis. In other embodiments, the WD peptide can be provided a fusion polypeptide with all or a portion of the antenopodia III protein.

To further illustrate, the WD peptide (or peptidomimetic) can be provided as a chimeric peptide which includes a heterologous peptide sequence ("internalizing peptide") which drives the translocation of an extracellular form of a WD peptide sequence across a cell membrane in order to facilitate intracellular localization of the WD peptide. In this regard, the therapeutic WD sequence is one which is active intracellularly. The internalizing peptide, by itself, is capable of crossing a cellular membrane by, e.g., transcytosis, at a relatively high rate.

The internalizing peptide is conjugated, e.g., as a fusion protein, to the WD peptide. The resulting chimeric peptide is transported into cells at a higher rate relative to the activator polypeptide alone to thereby provide a means for enhancing its introduction into cells to which it is applied, e.g., to enhance topical applications of the WD peptide.

In one embodiment, the internalizing peptide is derived from the *Drosophila* antennapedia protein, or homologs thereof. The 60 amino acid long homeodomain of the homeo-protein antennapedia has been demonstrated to translocate through biological membranes and can facilitate the translocation of heterologous polypeptides to which it is coupled. See for example Derossi et al. (1994) *J Biol Chem* 269:10444-10450; and Perez et al. (1992) *J Cell Sci* 102:717-722. Recently, it has been demonstrated that fragments as small as 16 amino acids long of this protein are sufficient to drive internalization. See Derossi et al. (1996) *J Biol Chem* 271:18188-18193.

The present invention contemplates a WD peptide or peptidomimetic sequence as described herein, and at least a portion of the Antennapedia protein (or homolog thereof) sufficient to increase the transmembrane transport of the chimeric protein, relative to the WD peptide or peptidomimetic, by a statistically significant amount.

Another example of an internalizing peptide is the HIV transactivator (TAT) protein. This protein appears to be divided into four domains (Kuppuswamy et al. (1989) *Nucl. Acids Res.* 17:3551-3561). Purified TAT protein is taken up by cells in tissue culture (Frankel and Pabo, (1989) *Cell* 55:1189-1193), and peptides, such as the fragment corresponding to residues 37 -62 of TAT, are rapidly taken up by cell in vitro (Green and Loewenstein, (1989) *Cell* 55:1179-1188). The highly basic region mediates internalization and targeting of the internalizing moiety to the nucleus (Ruben et al., (1989) *J. Virol.* 63:1-8).

Another exemplary transcellular polypeptide can be generated to include a sufficient portion of mastoparan (T. Higashijima et al., (1990) *J. Biol. Chem.* 265:14176) to increase the transmembrane transport of the chimeric protein.

While not wishing to be bound by any particular theory, it is noted that hydrophilic polypeptides may also be physiologically transported across the membrane barriers by coupling or conjugating the polypeptide to a transportable peptide which is capable of crossing the membrane by receptor-mediated transcytosis. Suitable internalizing peptides of this type can be generated using all or a portion of, e.g., a histone, insulin, transferrin, basic albumin, prolactin and insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II) or other growth factors. For instance, it has been found that an insulin fragment, showing affinity

for the insulin receptor on capillary cells, and being less effective than insulin in blood sugar reduction, is capable of transmembrane transport by receptor-mediated transcytosis and can therefor serve as an internalizing peptide for the subject transcellular peptides and peptidomimetics. Preferred growth factor-derived internalizing peptides include EGF (epidermal growth factor)-derived peptides, such as CMHIESLDSYTC and CMYIEALDKYAC; TGF- $\beta$  (transforming growth factor  $\beta$ )-derived peptides; peptides derived from PDGF (platelet-derived growth factor) or PDGF-2; peptides derived from IGF-I (insulin-like growth factor) or IGF-II; and FGF (fibroblast growth factor)-derived peptides.

Another class of translocating/internalizing peptides exhibits pH-dependent membrane binding. For an internalizing peptide that assumes a helical conformation at an acidic pH, the internalizing peptide acquires the property of amphiphilicity, e.g., it has both hydrophobic and hydrophilic interfaces. More specifically, within a pH range of approximately 5.0-5.5, an internalizing peptide forms an  $\alpha$ -helical, amphiphilic structure that facilitates insertion of the moiety into a target membrane. An  $\alpha$ -helix-inducing acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes. Such internalizing peptides can be used to facilitate transport of WD peptides and peptidomimetics, taken up by an endocytic mechanism, from endosomal compartments to the cytoplasm.

A preferred pH-dependent membrane-binding internalizing peptide includes a high percentage of helix-forming residues, such as glutamate, methionine, alanine and leucine. In addition, a preferred internalizing peptide sequence includes ionizable residues having pKa's within the range of pH 5-7, so that a sufficient uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane.

A particularly preferred pH-dependent membrane-binding internalizing peptide in this regard is aa1-aa2-aa3-EAALA(EALA)4-EALEALAA-amide, which represents a modification of the peptide sequence of Subbarao et al. (Biochemistry 26:2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue, such as cysteine or lysine, that facilitates chemical conjugation of the internalizing peptide to a targeting protein conjugate. Amino acid residues 2-3 may be selected to modulate the affinity of the internalizing peptide for different membranes. For instance, if both residues 2 and 3 are lys or arg, the internalizing peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the internalizing peptide will insert into neutral membranes.

Yet other preferred internalizing peptides include peptides of apo-lipoprotein A-1 and B; peptide toxins, such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid hormone, pancreatic polypeptide; and peptides corresponding to signal sequences of numerous secreted proteins. In addition, exemplary internalizing peptides may be modified through attachment of substituents that enhance the alpha-helical character of the internalizing peptide at acidic pH.

Yet another class of internalizing peptides suitable for use within the present invention include hydrophobic domains that are "hidden" at physiological pH, but are exposed in the low pH environment of the target cell endosome. Upon pH-induced unfolding and exposure of the hydrophobic domain, the moiety binds to lipid bilayers and effects translocation of the covalently linked polypeptide into the cell cytoplasm. Such internalizing peptides may be modeled after sequences identified in, e.g., *Pseudomonas* exotoxin A, clathrin, or Diphtheria toxin.

Pore-forming proteins or peptides may also serve as internalizing peptides herein. Pore-forming proteins or peptides may be obtained or derived from, for example, C9 complement protein, cytolytic T-cell molecules or NK-cell molecules. These moieties are capable of forming ring-like structures in membranes, thereby allowing transport of attached polypeptide through the membrane and into the cell interior.

Mere membrane intercalation of an internalizing peptide may be sufficient for translocation of the WD peptide or peptidomimetic, across cell membranes. However, translocation may be improved by attaching to the internalizing peptide a substrate for intracellular enzymes (i.e., an "accessory peptide"). It is preferred that an accessory peptide be attached to a portion(s) of the internalizing peptide that protrudes through the cell membrane to the cytoplasmic face. The accessory peptide may be advantageously attached to one terminus of a translocating/internalizing moiety or anchoring peptide. An accessory moiety of the present invention may contain one or more amino acid residues. In one embodiment, an accessory moiety may provide a substrate for cellular phosphorylation (for instance, the accessory peptide may contain a tyrosine residue).

An exemplary accessory moiety in this regard would be a peptide substrate for N-myristoyl transferase, such as GNAAAARR (Eubanks et al., in: *Peptides. Chemistry and Biology*, Garland Marshall (ed.), ESCOM, Leiden, 1988, pp. 566-69) In this construct, an internalizing peptide would be attached to the C-terminus of the accessory peptide, since the

N-terminal glycine is critical for the accessory moiety's activity. This hybrid peptide, upon attachment to a WD peptide or peptidomimetic at its C-terminus, is N-myristylated and further anchored to the target cell membrane, e.g., it serves to increase the local concentration of the peptide at the cell membrane.

To further illustrate use of an accessory peptide, a phosphorylatable accessory peptide is first covalently attached to the C-terminus of an internalizing peptide and then incorporated into a fusion protein with a WD peptide or peptidomimetic. The peptide component of the fusion protein intercalates into the target cell plasma membrane and, as a result, the accessory peptide is translocated across the membrane and protrudes into the cytoplasm of the target cell. On the cytoplasmic side of the plasma membrane, the accessory peptide is phosphorylated by cellular kinases at neutral pH. Once phosphorylated, the accessory peptide acts to irreversibly anchor the fusion protein into the membrane. Localization to the cell surface membrane can enhance the translocation of the polypeptide into the cell cytoplasm.

Suitable accessory peptides include peptides that are kinase substrates, peptides that possess a single positive charge, and peptides that contain sequences which are glycosylated by membrane-bound glycotransferases. Accessory peptides that are glycosylated by membrane-bound glycotransferases may include the sequence x-NLT-x, where "x" may be another peptide, an amino acid, coupling agent or hydrophobic molecule, for example. When this hydrophobic tripeptide is incubated with microsomal vesicles, it crosses vesicular membranes, is glycosylated on the luminal side, and is entrapped within the vesicles due to its hydrophilicity (C. Hirschberg et al., (1987) *Ann. Rev. Biochem.* 56:63-87). Accessory peptides that contain the sequence x-NLT-x thus will enhance target cell retention of corresponding polypeptide.

In another embodiment of this aspect of the invention, an accessory peptide can be used to enhance interaction of the WD peptide or peptidomimetic with the target cell. Exemplary accessory peptides in this regard include peptides derived from cell adhesion proteins containing the sequence "RGD", or peptides derived from laminin containing the sequence CDPGYIGSRC. Extracellular matrix glycoproteins, such as fibronectin and laminin, bind to cell surfaces through receptor-mediated processes. A tripeptide sequence, RGD, has been identified as necessary for binding to cell surface receptors. This sequence is present in fibronectin, vitronectin, C3bi of complement, von-Willebrand factor, EGF receptor, transforming growth factor beta, collagen type I, lambda receptor of E. Coli, fibrinogen and Sindbis coat protein (E. Ruoslahti, *Ann. Rev. Biochem.* 57:375-413, 1988). Cell surface receptors that recognize RGD sequences have been grouped into a superfamily of related proteins designated "integrins".



Binding of "RGD peptides" to cell surface integrins will promote cell-surface retention, and ultimately translocation, of the polypeptide.

As described above, the internalizing and accessory peptides can each, independently, be added to WD peptide or peptidomimetic by either chemical cross-linking or in the form of a fusion protein. In the instance of fusion proteins, unstructured polypeptide linkers can be included between each of the peptide moieties.

In general, the internalization peptide will be sufficient to also direct export of the polypeptide. However, where an accessory peptide is provided, such as an RGD sequence, it may be necessary to include a secretion signal sequence to direct export of the fusion protein from its host cell. In preferred embodiments, the secretion signal sequence is located at the extreme N-terminus, and is (optionally) flanked by a proteolytic site between the secretion signal and the rest of the fusion protein.

In an exemplary embodiment, a WD peptide or peptidomimetic is engineered to include an integrin-binding RGD peptide/SV40 nuclear localization signal (see, for example Hart SL et al., 1994; J. Biol. Chem., 269:12468-12474), such as encoded by the nucleotide sequence provided in the NdeI - EcoRI fragment: catatgggtggctgccgtggcatatgttcggttgccgtgctctcctcaaaaagaagagaaag-gtagctggattc, which encodes the RGD/SV40 nucleotide sequence: MGGCRGDMFGCGAPP-KKKRKVAGF. In another embodiment, the protein can be engineered with the HIV-1 tat(1-72) polypeptide, e.g., as provided by the NdeI - EcoRI fragment: catatggagccagtagatcctagactagagccc-tggaagcatccaggaagtcagcctaaaactgcttgtaccaattgct attgtaaaaagtgttgctttcattgccaaagttgtttcataacaaaagcccttgatcctctatggcaggaagaagcggagacagcgacg aagacctcctcaaggcagtcagactcatcaagtttctctaagtaagcaaggattc, which encodes the HIV-1 tat(1-72) peptide sequence: MEPVDPRLEPWKHPGSQPKT-ACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRR RPPQGSQTHQVSLSKQ. In still another embodiment, the fusion protein includes the HSV-1 VP22 polypeptide (Elliott G., O'Hare P (1997) Cell, 88:223-233) provided by the NdeI-EcoRI fragment:

cat atg acc tct cgc cgc tcc gtg aag tcg ggt ccg cgg gag gtt ccg cgc gat gag tac gag gat ctg tac tac acc ccg tct tca ggt atg gcg agt ccc gat agt ccg cct gac acc tcc cgc cgt ggc gcc cta cag aca cgc tcg cgc cag agg ggc gag gtc cgt ttc gtc cag tac gac gag tcg gat tat gcc ctc tac ggg ggc tcg tca tcc gaa gac gac gaa cac ccg gag gtc ccc cgg acg cgg cgt ccc gtt tcc ggg gcg gtt ttg tcc ggc ccg ggg cct gcg cgg gcg cct ccg cca ccc gct ggg tcc gga ggg gcc gga cgc aca ccc acc acc gcc ccc cgg gcc ccc cga

acc cag cgg gtg gcg act aag gcc ccc gcg gcc ccg gcg gcg gag acc acc cgc ggc agg aaa tcg gcc cag  
cca gaa tcc gcc gca ctc cca gac gcc ccc gcg tcg acg gcg cca acc cga tcc aag aca ccc gcg cag ggg  
ctg gcc aga aag ctg cac ttt agc acc gcc ccc cca aac ccc gac gcg cca tgg acc ccc cgg gtg gcc ggc  
ttt aac aag cgc gtc ttc tgc gcc gcg gtc ggg cgc ctg gcg gcc atg cat gcc cgg atg gcg gcg gtc cag ctc  
tgg gac atg tcg cgt ccg cgc aca gac gaa gac ctc aac gaa ctc ctt ggc atc acc acc atc cgc gtg acg gtc  
tgc gag ggc aaa aac ctg ctt cag cgc gcc aac gag ttg gtg aat cca gac gtg gtg cag gac gtc gac gcg gcc  
acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc  
ccc aga cgg ccc gtc gag gaa ttc

which encodes the HSV-1 VP22 peptide having the sequence:

MTSRRSVKSGPREVPRDEYEDLYYTPSSGMASPDSPDTSRRGALQTRSRQRGEVRF  
VQYDESDYALYGGSSSEDEHPEVPRTRRPVSGAVLSGPGPARAPPPAGSGGAGRT  
PTTAPRAPRTGRVATKAPAAPAAETTRGRKSAQPESAALPDAPASTAPTRSKTPAQG  
LARKLHFSTAPPNPDPWTPRVAGFNKRVFCAAVGRLAAMHARMAAVQLWDMR  
PRTDEDLNELLGITTRVTVCEGKNLLQRANELVNPVVQDVDAATATRGRSAASRP  
TERPRAPARSASRPRRPVE

In still another embodiment, the fusion protein includes the C-terminal domain of the VP22 protein from, e.g., the nucleotide sequence (NdeI-EcoRI fragment):

cat atg gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga  
gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the VP22 (C-terminal domain) peptide sequence:  
MDVDAATATRGRSA-ASRPTERPRAPARSASRPRRPVE

In certain instances, it may also be desirable to include a nuclear localization signal as part of the WD peptide.

In the generation of fusion polypeptides including the subject WD peptides, it may be necessary to include unstructured linkers in order to ensure proper folding of the various peptide domains, and prevent steric or other interference of the heterologous domains with the PV inhibitory activity of the WD peptide. Many synthetic and natural linkers are known in the art and can be adapted for use in the present invention, including the (Gly3Ser)<sub>4</sub> linker.

In other embodiments, the subject WD therapeutics are peptidomimetics of the WD peptide. Peptidomimetics are compounds based on, or derived from, peptides and proteins. The WD peptidomimetics of the present invention typically can be obtained by structural modification of a known WD peptide sequence using unnatural amino acids, conformational restraints, isosteric replacement, and the like. The subject peptidomimetics constitute the

continuum of structural space between peptides and non-peptide synthetic structures; WD peptidomimetics may be useful, therefore, in delineating pharmacophores and in helping to translate peptides into nonpeptide compounds with the activity of the parent WD peptides.

Moreover, as is apparent from the present disclosure, mimetopes of the subject WD peptides can be provided. Such peptidomimetics can have such attributes as being non-hydrolyzable (e.g., increased stability against proteases or other physiological conditions which degrade the corresponding peptide), increased specificity and/or potency for inhibition of PV replication, and increased cell permeability for intracellular localization of the peptidomimetic. For illustrative purposes, peptide analogs of the present invention can be generated using, for example, benzodiazepines (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans 1*:1231), b-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71), diaminoketones (Natarajan et al. (1984) *Biochem Biophys Res Commun* 124:141), and methyleneamino-modified (Roark et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

In addition to a variety of sidechain replacements which can be carried out to generate the subject WD peptidomimetics, the present invention specifically contemplates the use of conformationally restrained mimics of peptide secondary structure. Numerous surrogates have been developed for the amide bond of peptides. Frequently exploited surrogates for the amide bond include the following groups (i) trans-olefins, (ii) fluoroalkene, (iii) methyleneamino, (iv) phosphonamides, and (v) sulfonamides.

Additionally, peptidomimetics based on more substantial modifications of the backbone of the WD peptide can be used. Peptidomimetics which fall in this category include (i) retro-inverso analogs, and (ii) N-alkyl glycine analogs (so-called peptoids).

Furthermore, the methods of combinatorial chemistry are being brought to bear, e.g., by G.L. Verdine at Harvard University, on the development of new peptidomimetics. For example, one embodiment of a so-called "peptide morphing" strategy focuses on the random generation of a library of peptide analogs that comprise a wide range of peptide bond substitutes.

Such retro-inverso analogs can be made according to the methods known in the art, such as that described by the Sisto et al. U.S. Patent 4,522,752. For example, the illustrated retro-inverso analog can be generated as follows. The geminal diamine corresponding to the N-terminal tryptophan is synthesized by treating a protected tryptophan analog with ammonia under HOBT-DCC coupling conditions to yield the N-Boc amide, and then effecting a Hofmann-type rearrangement with I,I-bis-(trifluoroacetoxy)iodobenzene (TIB), as described in Radhakrishna et al. (1979) J. Org. Chem. 44:1746. The product amine salt is then coupled to a side-chain protected (e.g., as the benzyl ester) N-Fmoc D-lys residue under standard conditions to yield the pseudodipeptide. The Fmoc (fluorenylmethoxycarbonyl) group is removed with piperidine in dimethylformamide, and the resulting amine is trimethylsilylated with bistrimethylsilylacetamide (BSA) before condensation with suitably alkylated, side-chain protected derivative of Meldrum's acid, as described in U.S. Patent 5,061,811 to Pinori et al., to yield the retro-inverso tripeptide analog WKH. The pseudotripeptide is then coupled with with an L-methionine analog under standard conditions to give the protected tetrapeptide analog. The protecting groups are removed to release the product, and the steps repeated to enlogate the tetrapeptide to the full length peptidomimetic. It will be understood that a mixed peptide, e.g. including some normal peptide linkages, will be generated. As a general guide, sites which are most susceptible to proteolysis are typically altered, with less susceptible amide linkages being optional for mimetic switching. The final product, or intermediates thereof, can be purified by HPLC.

Retro-enantio analogs such as this can be synthesized commercially available D-amino acids (or analogs thereof) and standard solid- or solution-phase peptide-synthesis techniques. For example, in a preferred solid-phase synthesis method, a suitably amino-protected (t-butyloxycarbonyl, Boc) D-trp residue (or analog thereof) is covalently bound to a solid support such as chloromethyl resin. The resin is washed with dichloromethane (DCM), and the BOC protecting group removed by treatment with TFA in DCM. The resin is washed and neutralized, and the next Boc-protected D-amino acid (D-lys) is introduced by coupling with diisopropylcarbodiimide. The resin is again washed, and the cycle repeated for each of the remaining amino acids in turn (D-his, D-met, etc). When synthesis of the protected retro-enantio

peptide is complete, the protecting groups are removed and the peptide cleaved from the solid support by treatment with hydrofluoric acid/anisole/dimethyl sulfide/thioanisole. The final product is purified by HPLC to yield the pure retro-enantio analog.

The trans olefin analog of a WD peptide can be synthesized according to the method of Y.K. Shue et al. (1987) Tetrahedron Letters 28:3225. Referring to the illustrated example, Boc-amino L-Ile is converted to the corresponding  $\alpha$ -amino aldehyde, which is treated with a vinylcuprate to yield a diastereomeric mixture of alcohols, which are carried on together. The allylic alcohol is acetylated with acetic anhydride in pyridine, and the olefin is cleaved with osmium tetroxide/sodium periodate to yield the aldehyde, which is condensed with the Wittig reagent derived from a protected tyrosine precursor, to yield the allylic acetate. The allylic acetate is selectively hydrolyzed with sodium carbonate in methanol, and the allylic alcohol is treated with triphenylphosphine and carbon tetrabromide to yield the allylic bromide. This compound is reduced with zinc in acetic acid to give the transposed trans olefin as a mixture of diastereomers at the newly-formed center. The diastereomers are separated and the pseudodipeptide is obtained by selective transfer hydrogenolysis to unveil the free carboxylic acid.

The pseudodipeptide is then coupled at the C-terminus, according to the above example, with a suitably protected tyrosine residue, and at the N-terminus with a protected alanine residue, by standard techniques, to yield the protected tetrapeptide isostere A-I-Y-Y. The tetrapeptide is then further condensed with the olefinic tripeptide analog derived by similar means for Lys-Ala-Arg, and so forth to build up the full WD peptide. The protecting groups are then removed with strong acid to yield the desired peptide analog, which can be further purified by HPLC.

Other pseudodipeptides can be made by the method set forth above merely by substitution of the appropriate starting Boc amino acid and Wittig reagent. Variations in the procedure may be necessary according to the nature of the reagents used, but any such variations will be purely routine and will be obvious to one of skill in the art.

It is further possible couple the pseudodipeptides synthesized by the above method to other pseudodipeptides, to make peptide analogs with several olefinic functionalities in place of amide functionalities. For example, pseudodipeptides corresponding to Met-Arg or Tyr-Lys, etc. could be made and then coupled together by standard techniques to yield an analog of the WD peptide which has alternating olefinic bonds between residues.

The synthesis of such phosphonate derivatives can be adapted from known synthesis schemes. See, for example, Loots et al. in *Peptides: Chemistry and Biology*, (Escom Science Publishers, Leiden, 1988, p. 118); Petrillo et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium, Pierce Chemical Co. Rockland, IL, 1985).

Many other peptidomimetic structures are known in the art and can be readily adapted for use in the the subject WD peptidomimetics. To illustrate, the WD peptidomimetic may incorporate the 1-azabicyclo[4.3.0]nonane surrogate ( see Kim et al. (1997) *J. Org. Chem.* 62:2847), or an N-acyl piperazic acid (see Xi et al. (1998) *J. Am. Chem. Soc.* 120:80), or a 2-substituted piperazine moiety as a constrained amino acid analogue (see Williams et al. (1996) *J. Med. Chem.* 39:1345-1348). In still other embodiments, certain amino acid residues can be replaced with aryl and bi-aryl moieties, e.g., monocyclic or bicyclic aromatic or heteroaromatic nucleus, or a biaromatic, aromatic-heteroaromatic, or biheteroaromatic nucleus. The subject WD peptidomimetics can be optimized by, e.g., combinatorial synthesis techniques combined with such high throughput screening as described herein.

Moreover, other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of inhibiting E1-WD interaction. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling. the predicted mimetope structures can then be produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

According to another aspect of this invention, WD peptides and peptidomimetics may be administered directly to PV infected cells. Direct delivery of such WD therapeutics may be facilitated by formulation of the peptidyl compound in any pharmaceutically acceptable

dosage form, e.g., for delivery orally, intratumorally, peritumorally, interlesionally, intravenously, intramuscularly, subcutaneously, periolesionally, or (preferably) topical routes, to exert local therapeutic effects.

Topical administration of the therapeutic is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the agent to diffuse into the affected cells. Successful intracellular delivery of agents not naturally taken up by cells has been achieved by exploiting the natural process of intracellular membrane fusion, or by direct access of the cell's natural transport mechanisms which include endocytosis and pinocytosis (Duzgunes (1985) Subcellular Biochemistry 11:195-286). Such processes are also useful in the direct delivery and uptake of the subject WD peptides and peptidomimetic by papillomavirus-infected cells.

In one embodiment, the membrane barrier can be overcome by associating the WD protein in complexes with lipid formulations closely resembling the lipid composition of natural cell membranes. In particular, the subject WD peptidomimetics are encapsulated in liposomes to form pharmaceutical preparations suitable for administration to living cells and, in particular, suitable for topical administration to human skin. The Yarosh U.S. Patent 5,190,762 demonstrates that proteins can be delivered across the outer skin layer and into living cells, without receptor binding, by liposome encapsulation.

These lipids are able to fuse with the cell membranes on contact, and in the process, the associated WD peptidomimetic is delivered intracellularly. Lipid complexes can not only facilitate intracellular transfers by fusing with cell membranes but also by overcoming charge repulsions between the cell membrane and the molecule to be inserted. The lipids of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form hollow lipid vesicles, or liposomes, in aqueous systems. This property can be used to entrap the WD peptidomimetic within the liposomes.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Liposomes have been described in the art as in vivo delivery vehicles. The structure of various types of lipid aggregates varies, depending on composition and method of forming the aggregate. Such aggregates include liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, having particle sizes in the nanometer to micrometer range. Methods of making lipid aggregates are by now well-known in the art. For example, the liposomes may be made from natural and synthetic phospholipids, glycolipids, and other lipids and lipid congeners; cholesterol, cholesterol derivatives and other cholesterol congeners; charged species which impart a net charge to the membrane; reactive species which can react after liposome formation to link additional molecules to the liposome membrane; and other lipid soluble compounds which have chemical or biological activity.

In one embodiment, pH sensitive liposomes are a preferred type of liposome for use with the present invention. One pathway for the entry of liposomes into cellular cytoplasm is by endocytosis into lysosomes of low pH. Accordingly, liposomes which are stable at neutral pH but release their contents at acidic pH can be used to deliver enzymes into the lysosomes of the cytoplasm, whereupon the contents are released.

Liposomes can be made sensitive to the low pH of the lysosomes by the lipid composition. In particular, pH sensitive liposomes can be prepared by using phospholipids which form lipid bilayers when charged but fail to stack in an ordered fashion when neutralized. An example of such a phospholipid is phosphatidylethanolamine, which is negatively charged above pH 9. The net charge of a phospholipid can be maintained at a pH which would otherwise neutralize the head groups by including charged molecules in the lipid bilayer which themselves can become neutralized. Examples of these charged molecules are oleic acid and cholesteryl hemisuccinate, which are negatively charged at neutral pH but become neutralized at pH 5. The effect of combining these together in a lipid bilayer is that at pH 9 all molecules are charged; at pH 7 the net negative charge of the oleic acid and cholesteryl hemisuccinate maintains the stability of the phosphatidylethanolamine, and at pH 5 all components are protonated and the lipid membrane is destabilized. Additional neutral molecules, such as phosphatidylcholine, can be added to the liposomes as long as they do not interfere with stabilization of the pH sensitive phospholipid by the charged molecules.

In another embodiment, the WD peptidomimetic is formulated with a positively charged synthetic (cationic) lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles, which can fuse with the negatively charged lipids of the cell membranes of mammalian cells, resulting in uptake of the



contents of the liposome (see, for example, Felgner et al. (1987) PNAS 84:7413-7417; and U.S. Pat. No. 4,897,355 to Eppstein, D. et al.). Another cationic lipid which can be used to generate WD peptidomimetic containing liposomes is the DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethyl-ammonio)propane (DOTAP) in combination with a phospholipid to form delivery vesicles.

Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) and/or LipofectAMINE™, commercially available reagents, can be used to deliver the WD peptidomimetic directly into cells. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and can efficiently deliver functional WD peptidomimetic into, for example, keratinocytes. Sells et al. (1995) Biotechniques 19:72-76 describe a procedure for delivery of purified proteins into a variety of cells using such poycationic lipid preparations.

A significant body of information is emerging regarding the use of other cationic lipids for the delivery of macromolecules into cells. Other suitable lipid vesicles for direct delivery of the WD peptidomimetic include vesicles containing a quaternary ammonium surfactant (Ballas et al. (1988) Biochim. Biophys Acta 939:8-18); lipophilic derivatives of spermine (Behr et al. (1989) PNAS 86:6982-6986).

The lipid formulations of the subject WD peptidomimetic can be used in pharmaceutical formulations to deliver the WD peptidomimetic by various routes and to various sites in the animal body to achieve the desired therapeutic effect. Local or systemic delivery of the therapeutic agent can be achieved by administration comprising application or insertion of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, intradermal, peritoneal, subcutaneous and topical administration.

Topical formulations are those advantageously applied to the skin or mucosa. Target mucosa can be that of the vaginal, cervical, vulvar, penal or anorectal mucosa, or target mucosa can be that of the gastrointestinal tract, comprising the mouth, larynx, esophagous and stomach. Lipids present in topical formulations can act to facilitate introduction of therapeutic WD peptidomimetic into the target tissue, such as the stratum or corneum of the skin, by perturbing the barrier properties of the protective membrane, or by introducing perturbing agents or penetration enhancers such as DMSO, Azone™ or by promoting the activity of these penetration enhancers.

Other pharmaceutical formulations comprising the cationic lipids of the invention are topical preparations containing an anesthetic or cytostatic agent, immunomodulators, bioactive peptides or oligonucleotides, sunscreens or cosmetics. Preparations for topical use are conveniently prepared with hydrophilic and hydrophobic bases in the form of creams, lotions, ointments or gels; alternatively, the preparation may be in the form of a liquid that is sprayed on the skin. The effect of the cationic lipids is to facilitate the penetration of the active antiviral agent through the stratum corneum of the dermis.

The composition and form of pharmaceutical preparations comprising the liposome, in combination with the WD peptidomimetic, can vary according to the intended route of administration.

Also, by suitable modifications of the liposome membranes, the liposomes can be made to bind to specific sub-populations of cells.

In still another embodiment, the therapeutic WD peptidomimetic can be delivered by way of an artificial viral envelope (AVE). The art as described a number of viral envelopes which which exploit molecular recognition of cell surface receptors by viral surface proteins as a means for selective intracellular delivery of macromolecules, including proteins. According to the method of Schreier, et. al., U.S. Patent 5,252,348, a virtually unlimited number of artificial viral envelopes can be prepared and applied using recombinant or isolated surface determinants. For example, the AVEs be generated as viral mimetics of a number of human viruses including arboviruses; flaviviridae; bunyaviridae; hepatitis viruses; Epstein-Barr viruses; herpes viruses; paramyxoviruses; respiratory syncytical virus; retroviruses including human T-lymphotrophic virus type I and II (HTLV-I/II) and human immunodeficiency virus type 1 and 2 (HIV- 1/2 ); rhinoviruses; orthopoxviruses; and human papilloma viruses (particularly those engineered not to express E6 and/or E7).

In another embodiment, direct delivery of a therapeutic WD peptidomimetic may be facilitated by chemical modification of the polypeptide itself. One such modification involves increasing the lipophilicity of the WD peptidomimetic in order to increase binding to the cell surface, in turn, stimulating non-specific endocytosis of the protein. Lipophilicity may be increased by adding a lipophilic moiety (e.g., one or more fatty acid molecules) to the WD peptidomimetic. A wide variety of fatty acids may be employed. For example, the protein may be palmitoylated. Alternatively, a lipopeptide may be produced by fusion or cross-linking, to permit the WD peptidomimetic to resemble the natural lipopeptide from E.coli, tripalmitoyl-S-glycerylcysteinyl-serine, at its amino terminus. This lipopeptide has been

shown to increase the uptake of fused peptides (P. Hoffmann et al., (1988) Immunobiol. 177:158-70). Lipophilicity may also be increased by esterification of the protein at tyrosine residues or other amino acid residues. And uptake of the WD peptidomimetic may be increased by addition of a basic polymer such as polyarginine or polylysine (Shen et al. (1978) PNAS 75:1872-76).

Direct delivery of WD peptidomimetics according to this invention may also be effected by the use of transport moieties, such as protein carriers known to cross cell membranes. For example, a WD peptide may be fused to a carrier protein, preferably by a genetic fusion which may be expressed in a system such as E.coli, barulovirus or yeast. According to one embodiment of this invention, the amino terminus of the WD peptide may be fused to the carboxy terminus of a transport moiety using standard techniques.

Nucleotide sequences encoding such carrier-WD peptide fusion proteins, operatively linked to regulatory sequences, may be constructed and introduced into appropriate expression systems using conventional recombinant DNA procedures. The resulting fusion protein may then be purified and tested for its capacity to (1) enter intact eukaryotic cells and (2) inhibit viral DNA replication once inside the intact eukaryotic cells.

Useful carrier proteins include, for example, bacterial hemolysins or "blending agents", such as alamethicin or sulfhydryl activated lysins. Other carrier moieties which may be used include cell entry components of bacterial toxins, such as Pseudomonas exotoxin, tetanus toxin, ricin toxin, and diphtheria toxin. Also useful is melittin, from bee venom. Other useful carrier proteins include proteins which are viral receptors, cell receptors or cell ligands for specific receptors that are internalized, i.e., those which cross mammalian cell membranes via specific interaction with cell surface receptors, recognized and taken into the cell by cell surface receptors. Such cell ligands include, for example, epidermal growth factor, fibroblast growth factor, transferrin and platelet-derived growth factor. Alternatively, the ligand may be a non-peptide, such as mannose-6-phosphate, which permits internalization by the mannose-6-phosphate receptor. The transport moiety may also be selected from bacterial immunogens, parasitic immunogens, viral immunogens, immunoglobulins or fragments thereof that bind to target molecules, cytokines, growth factors, colony stimulating factors and hormones. A transport moiety may also be derived from the tat protein of HIV-1.

As an alternative or addition to the above-described chemical modifications and protein carriers, which may be employed alone or in combination, other agents which allow penetration of the keratinized cell layer may be employed to facilitate delivery of the WD

peptidomimetics of this invention to papillomavirus-infected cells. In topical applications, for example, the WD peptidomimetic may be administered in combination with dimethylsulfoxide, an agent which promotes penetration of cell membranes by substances mixed with it. Useful keratinolytic agents include, for example, salicylic acid, urea, and alpha -hydroxyacids. For such applications, the WD peptidomimetic and any other agent may be administered topically, in cream or gel form.

According to an alternate embodiment of this invention, the therapeutic WD peptidomimetic may be administered serially or in combination with other therapeutics used in the treatment of papillomavirus infections or diseases caused by them. Such therapeutics include interferons, such as IFN-g , IFN-a and IFN-b derived from natural sources or produced by recombinant techniques, other cell mediators formed by leukocytes or produced by recombinant techniques such as for example, interleukin-1, interleukin-2, tumor necrosis factor, macrophage colony stimulating factor, macrophage migration inhibitory factor, macrophage activation factor, lymphotoxin and fibroblast growth factor. Alternatively, the WD peptidomimetic may be administered serially or in combination with conventional therapeutic agents or regimens such as, for example, salicylic acid, podophyllotoxin, retinoic acid, surgery, laser therapy and cryotherapy. Such combination therapies may advantageously utilize less than conventional dosages of those agents, or involve less radical regimens, thus avoiding any potential toxicity or risks associated with those therapies.

It will also be understood by those skilled in the art that any of the above enumerated delivery methods may be augmented, where topical application is being carried out, by the use of ultrasound or iontophoretic delivery devices which facilitate transdermal delivery of proteins. See, for example, Banga et al. (1993) Pharm Res 10:697-702; and Mitragotri et al. (1995) Science 269:850-853.

In another aspect, the present invention relates to gene therapy constructs containing a nucleic acid encoding, for example in an exemplary method, a WD peptide of the present invention, operably linked to at least one transcriptional regulatory sequence. The gene constructs of the present invention are formulated to be used as a part of a gene therapy protocol to deliver the subject therapeutic protein to an animal to be treated.

Any of the methods known to the art for the insertion of DNA fragments into a vector may be used to construct expression vectors consisting of appropriate transcriptional/translational control signals and the desired WD peptide-encoding nucleotide sequence. See, for example, Maniatis T., Fritsch E.F., and Sambrook J. (1989): Molecular Cloning (A Laboratory

Manual), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and Ausubel F.M., Brent R., Kingston R.E., Moore, D.D., Seidman J.G., Smith J.A., and Struhl K. (1992): *Current Protocols in Molecular Biology*, John Wiley & Sons, New York. These methods may include in vitro DNA recombinant and synthetic techniques and in vivo genetic recombination. Expression of a nucleic acid sequence encoding a WD peptide may be regulated by a second nucleic acid sequence so that the peptide is expressed in a host infected or transfected with the recombinant DNA molecule. For example, expression of a WD peptide may be controlled by any promoter/enhancer element known in the art. The promoter activation may be tissue specific or inducible by a metabolic product or administered substance.

Promoters/enhancers which may be used to control the expression of the WD peptide in vivo include, but are not limited to, the native WD promoter, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human b-actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384), and Keratin gene promoters, such as Keratin 14.

Expression constructs of the subject WD peptides may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells in vivo. Approaches include insertion of the WD peptide coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO<sub>4</sub> precipitation carried out in vivo. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene

therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for in vivo introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid encoding the particular WD peptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., the recombinant WD peptide, are expressed efficiently in cells which have taken up viral vector nucleic acid.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a WD peptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the WD peptide-encoding gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic WD peptide coding sequence can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or "gene gun" techniques. In preferred embodiments, the gene therapy construct of the present invention is applied topically to an infected or transformed cells of the skin or mucosal tissue. A WD peptide gene construct can, in one embodiment, be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) Cancer Treat Rev 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene

delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

#### 4.7. Expression Vectors and Nucleic Acid Compositions

In another aspect of the invention, the proteins described herein are provided in expression vectors. For instance, expression vectors are contemplated which include a nucleotide sequence encoding a polypeptide containing a composite activator of the present invention, which coding sequence is operably linked to at least one transcriptional regulatory sequence. Regulatory sequences for directing expression of the instant fusion proteins are art-recognized and are selected by a number of well understood criteria. Exemplary regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding the fusion proteins of this invention. Such useful expression control sequences, include, for example, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, and the promoters of the yeast  $\bar{\gamma}$ -mating factors and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

As will be apparent, the subject gene constructs can be used to cause expression of the subject fusion proteins in cells propagated in culture, e.g. to produce proteins or polypeptides, including fusion proteins, for purification.

This invention also pertains to a host cell transfected with a recombinant gene in order to express one of the subject polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, a fusion proteins of the present invention may be expressed in bacterial cells such as E. coli, insect cells (baculovirus), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject fusion proteins - e.g., the target polypeptide:F-box protein chimeric polypeptides described herein. For example, a host cell transfected with an expression vector encoding a protein of interest can be cultured under appropriate conditions to allow expression of the protein to occur. The protein may be secreted, by inclusion of a secretion signal sequence, and isolated from a mixture of cells and medium containing the protein. Alternatively, the protein may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The proteins can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the protein.

Thus, a coding sequence for a fusion protein of the present invention can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures.

Expression vehicles for production of a recombinant protein include plasmids and other vectors. For instance, suitable vectors for the expression of the instant fusion proteins include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al., (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

The preferred mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived



vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant fusion proteins by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

In yet other embodiments, the subject expression constructs are derived by insertion of the subject gene into viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. As described in greater detail below, such embodiments of the subject expression constructs are specifically contemplated for use in various in vivo and ex vivo gene therapy protocols.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes in vivo, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding a

fusion protein of the present invention, e.g., a composite activator, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include  $\psi$ Crip,  $\psi$ Cre,  $\psi$ 2 and  $\psi$ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis et al., (1985) Science 230:1395-1398; Danos and Mulligan, (1988) PNAS USA 85:6460-6464; Wilson et al., (1988) PNAS USA 85:3014-3018; Armentano et al., (1990) PNAS USA 87:6141-6145; Huber et al., (1991) PNAS USA 88:8039-8043; Ferry et al., (1991) PNAS USA 88:8377-8381; Chowdhury et al., (1991) Science 254:1802-1805; van Beusechem et al., (1992) PNAS USA 89:7640-7644; Kay et al., (1992) Human Gene Therapy 3:641-647; Dai et al., (1992) PNAS USA 89:10892-10895; Hwu et al., (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al., (1989) PNAS USA 86:9079-9083; Julan et al., (1992) J. Gen Virol 73:3251-3255; and Goud et al., (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et al., (1991) J. Biol. Chem. 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) *BioTechniques* 6:616; Rosenfeld et al., (1991) *Science* 252:431-434; and Rosenfeld et al., (1992) *Cell* 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al., (1992) cited supra), endothelial cells (Lemarchand et al., (1992) *PNAS USA* 89:6482-6486), hepatocytes (Herz and Gerard, (1993) *PNAS USA* 90:2812-2816) and muscle cells (Quantin et al., (1992) *PNAS USA* 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) *Cell* 16:683; Berkner et al., supra; and Graham et al., in *Methods in Molecular Biology*, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted chimeric gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the viral E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject chimeric genes is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for

example Flotte et al., (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al., (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al., (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) *PNAS USA* 81:6466-6470; Tratschin et al., (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al., (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al., (1984) *J. Virol.* 51:611-619; and Flotte et al., (1993) *J. Biol. Chem.* 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistence of the recombinant gene in cells of the central nervous system and ocular tissue (Pepose et al., (1994) *Invest Ophthalmol Vis Sci* 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a protein in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding a composite activator can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al., (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with monoclonal antibodies against glioma-associated antigen (Mizuno et al., (1992) *Neurol. Med. Chir.* 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, any of the subject gene constructs can be used

to transfect specific cells in vivo using a soluble polynucleotide carrier comprising an antibody conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via -mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al., (1993) Science 260:926; Wagner et al., (1992) PNAS USA 89:7934; and Christiano et al., (1993) PNAS USA 90:2122).

In clinical settings, the gene delivery systems can be introduced into a patient by any of a number of methods, each of which is familiar in the art.

For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the construct in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al., (1994) PNAS USA 91: 3054-3057).

In some embodiments of the invention, the target gene to be regulated by the regulatable F-box protein is an endogenous gene, which contains an exogenous regulatable F-box protein sequence. The exogenous regulatable F-box protein sequence can be inserted into the endogenous gene's coding sequence. In certain embodiments, the endogenous target gene is a DNA binding protein, capable of binding with high affinity and specificity to a target sequence. In a preferred embodiment, the DNA binding protein is human. However, the DNA binding protein can be from any other species. For example, the DNA binding protein can be from the yeast GAL4 protein.

In other embodiments, the target gene to be regulated by the regulatable F-box protein is an exogenous gene. In some embodiments, the exogenous gene is integrated into the chromosomal DNA of a cell. The exogenous gene can be inserted into the chromosomal DNA, or the exogenous gene can substitute for at least a portion of an endogenous gene. Alternatively, the exogenous gene can be present on an extrachromosomal DNA element, such as a plasmid or a viral vector. The target gene can be present in a single copy or in multiple copies. In view of

the experimental results described herein, it is not necessary that the target gene be present in more than one copy. However, if even higher levels of protein encoded by the target gene is desired, multiple copies of the gene can be used.

A wide variety of genes can be employed as the target gene, including genes that encode a therapeutic protein. The target gene can be any sequence of interest which provides a desired phenotype. It can encode a surface membrane protein, a secreted protein, a cytoplasmic protein, or there can be a plurality of target genes encoding different products. The proteins which are expressed, singly or in combination, can involve homing, cytotoxicity, proliferation, immune response, inflammatory response, clotting or dissolving of clots, hormonal regulation, etc. The proteins expressed may be naturally-occurring proteins, mutants of naturally-occurring proteins, unique sequences, or combinations thereof.

Various secreted products include hormones, such as insulin, human growth hormone, glucagon, pituitary releasing factor, ACTH, melanotropin, relaxin, etc.; growth factors, such as EGF, IGF-1, TGF- $\beta$ ,  $\beta$ , PDGF, G-CSF, M-CSF, GM-CSF, FGF, erythropoietin, thrombopoietin, megakaryocytic stimulating and growth factors, etc.; interleukins, such as IL-1 to -13; TNF- $\beta$  and  $\beta$ , etc.; and enzymes and other factors, such as tissue plasminogen activator, members of the complement cascade, perforins, superoxide dismutase, coagulation factors, antithrombin-III, Factor VIIIc, Factor VIIIvW, Factor IX,  $\alpha$ -antitrypsin, proteinC, proteinS, endorphins, dynorphin, bone morphogenetic protein, CFTR, etc.

The gene can encode a naturally-occurring surface membrane protein or a protein made so by introduction of an appropriate signal peptide and transmembrane sequence. Various such proteins include homing receptors, e.g. L-selectin (Mel-14), blood-related proteins, particularly having a kringle structure, e.g. Factor VIIIc, Factor VIIIvW, hematopoietic cell markers, e.g. CD3, CD4, CD8, Bcell receptor, TCR subunits  $\beta$ ,  $\beta$ ,  $\beta$ ,  $\beta$ , CD10, CD19, CD28, CD33, CD38, CD41, etc., receptors, such as the interleukin receptors IL-2R, IL-4R, etc., channel proteins, for influx or efflux of ions, e.g. H<sup>+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, etc., and the like; CFTR, tyrosine activation motif, zap-70, etc.

Proteins may be modified for transport to a vesicle for exocytosis. By adding the sequence from a protein which is directed to vesicles, where the sequence is modified proximal to one or the other terminus, or situated in an analogous position to the protein source, the modified protein will be directed to the Golgi apparatus for packaging in a vesicle. This process in conjunction with the presence of the chimeric proteins for exocytosis allows for rapid transfer of the proteins to the extracellular medium and a relatively high localized concentration.

Also, intracellular proteins can be of interest, such as proteins in metabolic pathways, regulatory proteins, steroid receptors, transcription factors, etc., depending upon the nature of the host cell. Some of the proteins indicated above can also serve as intracellular proteins.

By way of further illustration, in T-cells, one may wish to introduce genes encoding one or both chains of a T-cell receptor. For B-cells, one could provide the heavy and light chains for an immunoglobulin for secretion. For cutaneous cells, e.g. keratinocytes, particularly stem cells keratinocytes, one could provide for protection against infection, by secreting  $\gamma$ -,  $\delta$ - or  $\epsilon$ -interferon, antichemotactic factors, proteases specific for bacterial cell wall proteins, etc.

In addition to providing for expression of a gene having therapeutic value, there will be many situations where one may wish to direct a cell to a particular site. The site can include anatomical sites, such as lymph nodes, mucosal tissue, skin, synovium, lung or other internal organs or functional sites, such as clots, injured sites, sites of surgical manipulation, inflammation, infection, etc. By providing for expression of surface membrane proteins which will direct the host cell to the particular site by providing for binding at the host target site to a naturally-occurring epitope, localized concentrations of a secreted product can be achieved. Proteins of interest include homing receptors, e.g. L-selectin, GMP140, CLAM-1, etc., or addressins, e.g. ELAM-1, PNA<sub>d</sub>, LNA<sub>d</sub>, etc., clot binding proteins, or cell surface proteins that respond to localized gradients of chemotactic factors. There are numerous situations where one would wish to direct cells to a particular site, where release of a therapeutic product could be of great value.

For use in gene therapy, the target gene can encode any gene product that is beneficial to a subject. The gene product can be a secreted protein, a membraneous protein, or a cytoplasmic protein. Preferred secreted proteins include growth factors, differentiation factors, cytokines, interleukins, tPA, and erythropoietin. Preferred membraneous proteins include receptors, e.g. growth factor or cytokine receptors or proteins mediating apoptosis, e.g., Fas receptor. Other candidate therapeutic genes are disclosed in PCT/US93/01617.

In yet another embodiment, a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous gene, can be used to introduce recognition elements for a DNA binding activity of one of the subject engineered proteins. A variety of different formats for the gene activation

constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

#### 4.8. Kits

This invention further provides kits useful for the foregoing applications. One such kit contains one or more nucleic acids encoding a chimeric polypeptide comprising a target polypeptide which encodes a bioactivity and a regulatable F-box protein, which is inserted into the target polypeptide. The kit may further comprise an additional nucleic acids such as specialized vectors which contain a cloning site for insertion of a desired target gene by the practitioner. For example, a preferred kit would contain a cloning site comprising at least one restriction site for insertion of an N-Target polypeptide of a target polypeptide, which is supplied by the user of the kit. In preferred embodiments, the cloning site is a polylinker. In preferred embodiments, this N-Target polypeptide cloning site is followed by a regulatable F-box protein sequence. In particularly preferred embodiments, the N-Target polypeptide cloning site of the vector is made available to the user in all three possible reading frames by supplying three different versions of the vector corresponding to single nucleotide insertions at the cloning site so that an in-frame fusion of the N-Target polypeptide to the regulatable F-box protein occurs. In preferred embodiments, the regulatable F-box protein sequence is further followed by a cloning site for a C-Target polypeptide element of the target sequence, which target may be supplied by the user. In still more preferred embodiments, versions of the vector corresponding to all three possible reading frames between the regulatable F-box protein and the C-target polypeptide are made available to the user. For regulatable applications, i.e., in cases in which the recombinant protein contains a ligand binding domain or inducible domain, the kit may further contain an oligomerizing agent, such as the macrolide dimerizers discussed above. Such kits may for example contain a sample of a dimerizing agent capable of dimerizing the two recombinant proteins and activating transcription of the target.

Constructs may be designed in accordance with the principles, illustrative examples and materials and methods disclosed in the patent documents and scientific literature cited herein, each of which is incorporated herein by reference, with modifications and further exemplification as described herein. Components of the constructs can be prepared in conventional ways, where the coding sequences and regulatory regions may be isolated, as appropriate, ligated, cloned in an appropriate cloning host, analyzed by restriction or sequencing, or other convenient means. Particularly, using PCR, individual fragments including all or



portions of a functional unit may be isolated, where one or more mutations may be introduced using "primer repair", ligation, *in vitro* mutagenesis, etc. as appropriate. In the case of DNA constructs encoding chimeric proteins, DNA sequences encoding individual domains and sub-domains are joined such that they constitute a single open reading frame encoding a chimeric protein capable of being translated in cells or cell lysates into a single polypeptide harboring all component domains. The DNA construct encoding the chimeric protein may then be placed into a vector that directs the expression of the protein in the appropriate cell type(s). For biochemical analysis of the encoded chimera, it may be desirable to construct plasmids that direct the expression of the protein in bacteria or in reticulocyte-lysate systems. For use in the production of proteins in mammalian cells, the protein-encoding sequence is introduced into an expression vector that directs expression in these cells. Expression vectors suitable for such uses are well known in the art. Various sorts of such vectors are commercially available.

#### 4.9. Transgenic Organisms

The invention provides transgenic plants and animals which carry one or more F-box protein modified target genes which can be regulated. These transgenic organisms can be generated with the nucleic acid target gene:F-box protein hybrids of the invention. For example, the invention further provides for transgenic animals, which can be used for a variety of purposes, e.g., to study the function of a target gene. The transgenic animals of the invention can be animals expressing a transgene encoding a target:F-box protein hybrid protein or fragment thereof or variants thereof, including mutants and polymorphic variants thereof. These animals can be used to determine the effect of expression of a target gene protein in a specific site or in a specific temporal window. In one aspect, the invention features a cell or cell line, which contains a knock-in of an F-box protein which has been inserted into a particular target gene. In a preferred embodiment, the cell or cell line is an undifferentiated cell, for example, a stem cell, embryonic stem cell, oocyte or embryonic cell.

Yet in a further aspect, the invention features a method of producing a non-human mammal with a targeted disruption in an interleukin-1 gene. For example, a target gene knock-in construct can be created with a portion of the target gene having an internal portion of said target gene replaced by a marker. The knock-out construct can then be transfected into a population of embryonic stem m(ES) cells. Transfected cells can then be selected as expressing the marker. The transfected ES cells can then be introduced into an embryo of an ancestor of said mammal. The embryo can be allowed to develop to term to produce a chimeric mammal

with the knock-out construct in its germline. Breeding said chimeric mammal will produce a heterozygous mammal with a targeted disruption in the target gene. Homozygotes can be generated by crossing heterozygotes.

In another aspect, the invention features target knock-out constructs, which can be used to generate the animals described above. In one embodiment, the target construct can comprise a portion of the target gene, wherein an internal portion of said target gene is replaced by a selectable marker. Preferably, the marker is the *neo* gene and the portion of the target gene is at least 2.5 kb long or 7.0 or 9.5 kb long (including the replaced portion and any target flanking sequences). The internal portion preferably covers at least a portion of an exon and in some embodiments it covers all of the exons which encode an target polypeptide.

Yet other non-human animals within the scope of the invention include those in which the expression of the endogenous Target gene has been mutated or "knocked out". A "knock out" animal is one carrying a homozygous or heterozygous deletion of a particular gene or genes. These animals could be useful to determine whether the absence of the target polypeptide will result in a specific phenotype, in particular whether these mice have or are likely to develop a specific disease, such as high susceptibility to heart disease or cancer. Furthermore these animals are useful in screens for drugs which alleviate or attenuate the disease condition resulting from the mutation of the target gene as outlined below. These animals are also useful for determining the effect of a specific amino acid difference, or allelic variation, in a target gene.

In a preferred embodiment of this aspect of the invention, a transgenic target gene knock-in mouse, carrying the mutated target locus on one or both of its chromosomes, is used as a model system for transgenic or drug treatment of the condition resulting from loss of target gene expression.

Methods for obtaining transgenic and knockout non-human animals are well known in the art. Knock out mice are generated by homologous integration of a "knock out" construct into a mouse embryonic stem cell chromosome which encodes the gene to be knocked out. In one embodiment, gene targeting, which is a method of using homologous recombination to modify an animal's genome, can be used to introduce changes into cultured embryonic stem cells. By targeting a specific gene of interest in ES cells, these changes can be introduced into the germlines of animals to generate chimeras. The gene targeting procedure is accomplished by introducing into tissue culture cells a DNA targeting construct that includes a segment homologous to a target locus, and which also includes an intended sequence modification to the

target genomic sequence (e.g., insertion, deletion, point mutation). The treated cells are then screened for accurate targeting to identify and isolate those which have been properly targeted.

Gene targeting in embryonic stem cells is in fact a scheme contemplated by the present invention as a means for disrupting a target gene function through the use of a targeting transgene construct designed to undergo homologous recombination with one or more target genomic sequences. The targeting construct can be arranged so that, upon recombination with an element of a gene, a positive selection marker is inserted into (or replaces) coding sequences of the gene. The inserted sequence functionally disrupts the target gene, while also providing a positive selection trait. Exemplary targeting constructs are described in more detail below.

Generally, the embryonic stem cells (ES cells) used to produce the knockout animals will be of the same species as the knockout animal to be generated. Thus for example, mouse embryonic stem cells will usually be used for generation of knockout mice.

Embryonic stem cells are generated and maintained using methods well known to the skilled artisan such as those described by Doetschman et al. (1985) *J. Embryol. Exp. Morphol.* 87:27-45). Any line of ES cells can be used, however, the line chosen is typically selected for the ability of the cells to integrate into and become part of the germ line of a developing embryo so as to create germ line transmission of the knockout construct. Thus, any ES cell line that is believed to have this capability is suitable for use herein. One mouse strain that is typically used for production of ES cells, is the 129J strain. Another ES cell line is murine cell line D3 (American Type Culture Collection, catalog no. CKL 1934) Still another preferred ES cell line is the WW6 cell line (Ioffe et al. (1995) *PNAS* 92:7357-7361). The cells are cultured and prepared for knockout construct insertion using methods well known to the skilled artisan, such as those set forth by Robertson in: *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. IRL Press, Washington, D.C. [1987]); by Bradley et al. (1986) *Current Topics in Devel. Biol.* 20:357-371); and by Hogan et al. (*Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1986]).

A knock out construct refers to a uniquely configured fragment of nucleic acid which is introduced into a stem cell line and allowed to recombine with the genome at the chromosomal locus of the gene of interest to be mutated. Thus a given knock out construct is specific for a given gene to be targeted for disruption. Nonetheless, many common elements exist among these constructs and these elements are well known in the art. A typical knock out construct contains nucleic acid fragments of not less than about 0.5 kb nor more than about 10.0

kb from both the 5' and the 3' ends of the genomic locus which encodes the gene to be mutated. These two fragments are separated by an intervening fragment of nucleic acid which encodes a positive selectable marker, such as the neomycin resistance gene (neo<sup>R</sup>). The resulting nucleic acid fragment, consisting of a nucleic acid from the extreme 5' end of the genomic locus linked to a nucleic acid encoding a positive selectable marker which is in turn linked to a nucleic acid from the extreme 3' end of the genomic locus of interest, omits most of the coding sequence for target or other gene of interest to be knocked out. When the resulting construct recombines homologously with the chromosome at this locus, it results in the loss of the omitted coding sequence, otherwise known as the structural gene, from the genomic locus. A stem cell in which such a rare homologous recombination event has taken place can be selected for by virtue of the stable integration into the genome of the nucleic acid of the gene encoding the positive selectable marker and subsequent selection for cells expressing this marker gene in the presence of an appropriate drug (neomycin in this example).

Variations on this basic technique also exist and are well known in the art. For example, a "knock-in" construct refers to the same basic arrangement of a nucleic acid encoding a 5' genomic locus fragment linked to nucleic acid encoding a positive selectable marker which in turn is linked to a nucleic acid encoding a 3' genomic locus fragment, but which differs in that none of the coding sequence is omitted and thus the 5' and the 3' genomic fragments used were initially contiguous before being disrupted by the introduction of the nucleic acid encoding the positive selectable marker gene. This "knock-in" type of construct is thus very useful for the construction of mutant transgenic animals when only a limited region of the genomic locus of the gene to be mutated, such as a single exon, is available for cloning and genetic manipulation. Alternatively, the "knock-in" construct can be used to specifically eliminate a single functional domain of the targeted gene, resulting in a transgenic animal which expresses a polypeptide of the targeted gene which is defective in one function, while retaining the function of other domains of the encoded polypeptide. This type of "knock-in" mutant frequently has the characteristic of a so-called "dominant negative" mutant because, especially in the case of proteins which homomultimerize, it can specifically block the action of (or "poison") the polypeptide product of the wild-type gene from which it was derived. In a variation of the knock-in technique, a marker gene is integrated at the genomic locus of interest such that expression of the marker gene comes under the control of the transcriptional regulatory elements of the targeted gene. A marker gene is one that encodes an enzyme whose activity can be detected (e.g., b-galactosidase), the enzyme substrate can be added to the cells under suitable

conditions, and the enzymatic activity can be analyzed. One skilled in the art will be familiar with other useful markers and the means for detecting their presence in a given cell. All such markers are contemplated as being included within the scope of the teaching of this invention.

As mentioned above, the homologous recombination of the above described "knock out" and "knock in" constructs is very rare and frequently such a construct inserts nonhomologously into a random region of the genome where it has no effect on the gene which has been targeted for deletion, and where it can potentially recombine so as to disrupt another gene which was otherwise not intended to be altered. Such nonhomologous recombination events can be selected against by modifying the abovementioned knock out and knock in constructs so that they are flanked by negative selectable markers at either end (particularly through the use of two allelic variants of the thymidine kinase gene, the polypeptide product of which can be selected against in expressing cell lines in an appropriate tissue culture medium well known in the art - i.e. one containing a drug such as 5-bromodeoxyuridine). Thus a preferred embodiment of such a knock out or knock in construct of the invention consist of a nucleic acid encoding a negative selectable marker linked to a nucleic acid encoding a 5' end of a genomic locus linked to a nucleic acid of a positive selectable marker which in turn is linked to a nucleic acid encoding a 3' end of the same genomic locus which in turn is linked to a second nucleic acid encoding a negative selectable marker. Nonhomologous recombination between the resulting knock out construct and the genome will usually result in the stable integration of one or both of these negative selectable marker genes and hence cells which have undergone nonhomologous recombination can be selected against by growth in the appropriate selective media (e.g. media containing a drug such as 5-bromodeoxyuridine for example). Simultaneous selection for the positive selectable marker and against the negative selectable marker will result in a vast enrichment for clones in which the knock out construct has recombined homologously at the locus of the gene intended to be mutated. The presence of the predicted chromosomal alteration at the targeted gene locus in the resulting knock out stem cell line can be confirmed by means of Southern blot analytical techniques which are well known to those familiar in the art. Alternatively, PCR can be used.

Each knockout construct to be inserted into the cell must first be in the linear form. Therefore, if the knockout construct has been inserted into a vector (described *infra*), linearization is accomplished by digesting the DNA with a suitable restriction endonuclease selected to cut only within the vector sequence and not within the knockout construct sequence.

For insertion, the knockout construct is added to the ES cells under appropriate conditions for the insertion method chosen, as is known to the skilled artisan. For example, if the ES cells are to be electroporated, the ES cells and knockout construct DNA are exposed to an electric pulse using an electroporation machine and following the manufacturer's guidelines for use. After electroporation, the ES cells are typically allowed to recover under suitable incubation conditions. The cells are then screened for the presence of the knock out construct as explained above. Where more than one construct is to be introduced into the ES cell, each knockout construct can be introduced simultaneously or one at a time.

After suitable ES cells containing the knockout construct in the proper location have been identified by the selection techniques outlined above, the cells can be inserted into an embryo. Insertion may be accomplished in a variety of ways known to the skilled artisan, however a preferred method is by microinjection. For microinjection, about 10-30 cells are collected into a micropipet and injected into embryos that are at the proper stage of development to permit integration of the foreign ES cell containing the knockout construct into the developing embryo. For instance, the transformed ES cells can be microinjected into blastocytes. The suitable stage of development for the embryo used for insertion of ES cells is very species dependent, however for mice it is about 3.5 days. The embryos are obtained by perfusing the uterus of pregnant females. Suitable methods for accomplishing this are known to the skilled artisan, and are set forth by, e.g., Bradley et al. (*supra*).

While any embryo of the right stage of development is suitable for use, preferred embryos are male. In mice, the preferred embryos also have genes coding for a coat color that is different from the coat color encoded by the ES cell genes. In this way, the offspring can be screened easily for the presence of the knockout construct by looking for mosaic coat color (indicating that the ES cell was incorporated into the developing embryo). Thus, for example, if the ES cell line carries the genes for white fur, the embryo selected will carry genes for black or brown fur.

After the ES cell has been introduced into the embryo, the embryo may be implanted into the uterus of a pseudopregnant foster mother for gestation. While any foster mother may be used, the foster mother is typically selected for her ability to breed and reproduce well, and for her ability to care for the young. Such foster mothers are typically prepared by mating with vasectomized males of the same species. The stage of the pseudopregnant foster mother is important for successful implantation, and it is species dependent. For mice, this stage is about 2-3 days pseudopregnant.

Offspring that are born to the foster mother may be screened initially for mosaic coat color where the coat color selection strategy (as described above, and in the appended examples) has been employed. In addition, or as an alternative, DNA from tail tissue of the offspring may be screened for the presence of the knockout construct using Southern blots and/or PCR as described above. Offspring that appear to be mosaics may then be crossed to each other, if they are believed to carry the knockout construct in their germ line, in order to generate homozygous knockout animals. Homozygotes may be identified by Southern blotting of equivalent amounts of genomic DNA from mice that are the product of this cross, as well as mice that are known heterozygotes and wild type mice.

Other means of identifying and characterizing the knockout offspring are available. For example, Northern blots can be used to probe the mRNA for the presence or absence of transcripts encoding either the gene knocked out, the marker gene, or both. In addition, Western blots can be used to assess the level of expression of the target gene knocked out in various tissues of the offspring by probing the Western blot with an antibody against the particular target protein, or an antibody against the marker gene product, where this gene is expressed. Finally, *in situ* analysis (such as fixing the cells and labeling with antibody) and/or FACS (fluorescence activated cell sorting) analysis of various cells from the offspring can be conducted using suitable antibodies to look for the presence or absence of the knockout construct gene product.

Yet other methods of making knock-out or disruption transgenic animals are also generally known. See, for example, *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Recombinase dependent knockouts can also be generated, e.g. by homologous recombination to insert target sequences, such that tissue specific and/or temporal control of inactivation of a Target-gene can be controlled by recombinase sequences (described *infra*).

Animals containing more than one knockout construct and/or more than one transgene expression construct are prepared in any of several ways. The preferred manner of preparation is to generate a series of mammals, each containing one of the desired transgenic phenotypes. Such animals are bred together through a series of crosses, backcrosses and selections, to ultimately generate a single animal containing all desired knockout constructs and/or expression constructs, where the animal is otherwise congenic (genetically identical) to the wild type except for the presence of the knockout construct(s) and/or transgene(s).

A targetted transgene can encode the wild-type form of the protein, or can encode homologs thereof, including both agonists and antagonists, as well as antisense constructs. In preferred embodiments, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, cis-acting sequences that control expression in the desired pattern. In the present invention, such mosaic expression of a target protein can be essential for many forms of lineage analysis and can additionally provide a means to assess the effects of, for example, lack of target gene expression which might grossly alter development in small patches of tissue within an otherwise normal embryo. Toward this end, tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

Genetic techniques, which allow for the expression of transgenes can be regulated via site-specific genetic manipulation *in vivo*, are known to those skilled in the art. For instance, genetic systems are available which allow for the regulated expression of a recombinase that catalyzes the genetic recombination of a target sequence. As used herein, the phrase "target sequence" refers to a nucleotide sequence that is genetically recombined by a recombinase. The target sequence is flanked by recombinase recognition sequences and is generally either excised or inverted in cells expressing recombinase activity. Recombinase catalyzed recombination events can be designed such that recombination of the target sequence results in either the activation or repression of expression of one of the subject target proteins. For example, excision of a target sequence which interferes with the expression of a recombinant target gene, such as one which encodes an antagonistic homolog or an antisense transcript, can be designed to activate expression of that gene. This interference with expression of the protein can result from a variety of mechanisms, such as spatial separation of the target gene from the promoter element or an internal stop codon. Moreover, the transgene can be made wherein the coding sequence of the gene is flanked by recombinase recognition sequences and is initially transfected into cells in a 3' to 5' orientation with respect to the promoter element. In such an instance, inversion of the target sequence will reorient the subject gene by placing the 5' end of the coding sequence in an orientation with respect to the promoter element which allow for promoter driven transcriptional activation.

The transgenic animals of the present invention all include within a plurality of their cells a transgene of the present invention, which transgene alters the phenotype of the "host



cell" with respect to regulation of cell growth, death and/or differentiation. Since it is possible to produce transgenic organisms of the invention utilizing one or more of the transgene constructs described herein, a general description will be given of the production of transgenic organisms by referring generally to exogenous genetic material. This general description can be adapted by those skilled in the art in order to incorporate specific transgene sequences into organisms utilizing the methods and materials described below.

In an illustrative embodiment, either the cre/loxP recombinase system of bacteriophage P1 (Lakso et al. (1992) PNAS 89:6232-6236; Orban et al. (1992) PNAS 89:6861-6865) or the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) Science 251:1351-1355; PCT publication WO 92/15694) can be used to generate *in vivo* site-specific genetic recombination systems. Cre recombinase catalyzes the site-specific recombination of an intervening target sequence located between loxP sequences. loxP sequences are 34 base pair nucleotide repeat sequences to which the Cre recombinase binds and are required for Cre recombinase mediated genetic recombination. The orientation of loxP sequences determines whether the intervening target sequence is excised or inverted when Cre recombinase is present (Abremski et al. (1984) J. Biol. Chem. 259:1509-1514); catalyzing the excision of the target sequence when the loxP sequences are oriented as direct repeats and catalyzes inversion of the target sequence when loxP sequences are oriented as inverted repeats.

Accordingly, genetic recombination of the target sequence is dependent on expression of the Cre recombinase. Expression of the recombinase can be regulated by promoter elements which are subject to regulatory control, e.g., tissue-specific, developmental stage-specific, inducible or repressible by externally added agents. This regulated control will result in genetic recombination of the target sequence only in cells where recombinase expression is mediated by the promoter element. Thus, the activation expression of a recombinant target protein can be regulated via control of recombinase expression.

Use of the cre/loxP recombinase system to regulate expression of a recombinant target protein requires the construction of a transgenic animal containing transgenes encoding both the Cre recombinase and the subject protein. Animals containing both the Cre recombinase and a recombinant target gene can be provided through the construction of "double" transgenic animals. A convenient method for providing such animals is to mate two transgenic animals each containing a transgene, e.g., a target gene and recombinase gene.

One advantage derived from initially constructing transgenic animals containing a target transgene in a recombinase-mediated expressible format derives from the likelihood that

the subject protein, whether agonistic or antagonistic, can be deleterious upon expression in the transgenic animal. In such an instance, a founder population, in which the subject transgene is silent in all tissues, can be propagated and maintained. Individuals of this founder population can be crossed with animals expressing the recombinase in, for example, one or more tissues and/or a desired temporal pattern. Thus, the creation of a founder population in which, for example, an antagonistic target transgene is silent will allow the study of progeny from that founder in which disruption of target mediated induction in a particular tissue or at certain developmental stages would result in, for example, a lethal phenotype.

Similar conditional transgenes can be provided using prokaryotic promoter sequences which require prokaryotic proteins to be simultaneously expressed in order to facilitate expression of the target transgene. Exemplary promoters and the corresponding trans-activating prokaryotic proteins are given in U.S. Patent No. 4,833,080.

Moreover, expression of the conditional transgenes can be induced by gene therapy-like methods wherein a gene encoding the trans-activating protein, e.g. a recombinase or a prokaryotic protein, is delivered to the tissue and caused to be expressed, such as in a cell-type specific manner. By this method, a target gene:F-box protein transgene could remain silent into adulthood until "turned on" by the introduction of the trans-activator.

In an exemplary embodiment, the "transgenic non-human animals" of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used to practice this invention are selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness. In addition, the haplotype is a significant factor. For example, when transgenic mice are to be produced, strains such as C57BL/6 or FVB lines are often used (Jackson Laboratory, Bar Harbor, ME). Preferred strains are those with H-2<sup>b</sup>, H-2<sup>d</sup> or H-2<sup>q</sup> haplotypes such as C57BL/6 or DBA/1. The line(s) used to practice this invention may themselves be transgenics, and/or may be knockouts (i.e., obtained from animals which have one or more genes partially or completely suppressed). In one embodiment, the transgene construct is introduced into a single stage embryo. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before

the first cleavage (Brinster et al. (1985) *PNAS* 82:4438-4442). As a consequence, all cells of the transgenic animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

Normally, fertilized embryos are incubated in suitable media until the pronuclei appear. At about this time, the nucleotide sequence comprising the transgene is introduced into the female or male pronucleus as described below. In some species such as mice, the male pronucleus is preferred. It is most preferred that the exogenous genetic material be added to the male DNA complement of the zygote prior to its being processed by the ovum nucleus or the zygote female pronucleus. It is thought that the ovum nucleus or female pronucleus release molecules which affect the male DNA complement, perhaps by replacing the protamines of the male DNA with histones, thereby facilitating the combination of the female and male DNA complements to form the diploid zygote.

Thus, it is preferred that the exogenous genetic material be added to the male complement of DNA or any other complement of DNA prior to its being affected by the female pronucleus. For example, the exogenous genetic material is added to the early male pronucleus, as soon as possible after the formation of the male pronucleus, which is when the male and female pronuclei are well separated and both are located close to the cell membrane. Alternatively, the exogenous genetic material could be added to the nucleus of the sperm after it has been induced to undergo decondensation. Sperm containing the exogenous genetic material can then be added to the ovum or the decondensed sperm could be added to the ovum with the transgene constructs being added as soon as possible thereafter.

Introduction of the transgene nucleotide sequence into the embryo may be accomplished by any means known in the art such as, for example, microinjection, electroporation, or lipofection. Following introduction of the transgene nucleotide sequence into the embryo, the embryo may be incubated in vitro for varying amounts of time, or reimplanted into the surrogate host, or both. In vitro incubation to maturity is within the scope of this invention. One common method is to incubate the embryos in vitro for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

For the purposes of this invention a zygote is essentially the formation of a diploid cell which is capable of developing into a complete organism. Generally, the zygote will be comprised of an egg containing a nucleus formed, either naturally or artificially, by the fusion of two haploid nuclei from a gamete or gametes. Thus, the gamete nuclei must be ones which are

naturally compatible, i.e., ones which result in a viable zygote capable of undergoing differentiation and developing into a functioning organism. Generally, a euploid zygote is preferred. If an aneuploid zygote is obtained, then the number of chromosomes should not vary by more than one with respect to the euploid number of the organism from which either gamete originated.

In addition to similar biological considerations, physical ones also govern the amount (e.g., volume) of exogenous genetic material which can be added to the nucleus of the zygote or to the genetic material which forms a part of the zygote nucleus. If no genetic material is removed, then the amount of exogenous genetic material which can be added is limited by the amount which will be absorbed without being physically disruptive. Generally, the volume of exogenous genetic material inserted will not exceed about 10 picoliters. The physical effects of addition must not be so great as to physically destroy the viability of the zygote. The biological limit of the number and variety of DNA sequences will vary depending upon the particular zygote and functions of the exogenous genetic material and will be readily apparent to one skilled in the art, because the genetic material, including the exogenous genetic material, of the resulting zygote must be biologically capable of initiating and maintaining the differentiation and development of the zygote into a functional organism.

The number of copies of the transgene constructs which are added to the zygote is dependent upon the total amount of exogenous genetic material added and will be the amount which enables the genetic transformation to occur. Theoretically only one copy is required; however, generally, numerous copies are utilized, for example, 1,000-20,000 copies of the transgene construct, in order to insure that one copy is functional. As regards the present invention, there will often be an advantage to having more than one functioning copy of each of the inserted exogenous DNA sequences to enhance the phenotypic expression of the exogenous DNA sequences.

Any technique which allows for the addition of the exogenous genetic material into nucleic genetic material can be utilized so long as it is not destructive to the cell, nuclear membrane or other existing cellular or genetic structures. The exogenous genetic material is preferentially inserted into the nucleic genetic material by microinjection. Microinjection of cells and cellular structures is known and is used in the art.

Reimplantation is accomplished using standard methods. Usually, the surrogate host is anesthetized, and the embryos are inserted into the oviduct. The number of embryos

implanted into a particular host will vary by species, but will usually be comparable to the number of off spring the species naturally produces.

Transgenic offspring of the surrogate host may be screened for the presence and/or expression of the transgene by any suitable method. Screening is often accomplished by Southern blot or Northern blot analysis, using a probe that is complementary to at least a portion of the transgene. Western blot analysis using an antibody against the protein encoded by the transgene may be employed as an alternative or additional method for screening for the presence of the transgene product. Typically, DNA is prepared from tail tissue and analyzed by Southern analysis or PCR for the transgene. Alternatively, the tissues or cells believed to express the transgene at the highest levels are tested for the presence and expression of the transgene using Southern analysis or PCR, although any tissues or cell types may be used for this analysis.

Alternative or additional methods for evaluating the presence of the transgene include, without limitation, suitable biochemical assays such as enzyme and/or immunological assays, histological stains for particular marker or enzyme activities, flow cytometric analysis, and the like. Analysis of the blood may also be useful to detect the presence of the transgene product in the blood, as well as to evaluate the effect of the transgene on the levels of various types of blood cells and other blood constituents.

Progeny of the transgenic animals may be obtained by mating the transgenic animal with a suitable partner, or by in vitro fertilization of eggs and/or sperm obtained from the transgenic animal. Where mating with a partner is to be performed, the partner may or may not be transgenic and/or a knockout; where it is transgenic, it may contain the same or a different transgene, or both. Alternatively, the partner may be a parental line. Where in vitro fertilization is used, the fertilized embryo may be implanted into a surrogate host or incubated in vitro, or both. Using either method, the progeny may be evaluated for the presence of the transgene using methods described above, or other appropriate methods.

The transgenic animals produced in accordance with the present invention will include exogenous genetic material. As set out above, the exogenous genetic material will, in certain embodiments, be a DNA sequence which results in the production of a target protein (either agonistic or antagonistic), and antisense transcript, or a target mutant. Further, in such embodiments the sequence will be attached to a transcriptional control element, e.g., a promoter, which preferably allows the expression of the transgene product in a specific type of cell.

Retroviral infection can also be used to introduce transgene into a non-human animal. The developing non-human embryo can be cultured in vitro to the blastocyst stage.

During this time, the blastomeres can be targets for retroviral infection (Jaenich, R. (1976) PNAS 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Manipulating the Mouse Embryo, Hogan eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al. (1985) PNAS 82:6927-6931; Van der Putten et al. (1985) PNAS 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart et al. (1987) EMBO J. 6:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al. (1982) Nature 298:623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line by intrauterine retroviral infection of the midgestation embryo (Jahner et al. (1982) supra).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro and fused with embryos (Evans et al. (1981) Nature 292:154-156; Bradley et al. (1984) Nature 309:255-258; Gossler et al. (1986) PNAS 83: 9065-9069; and Robertson et al. (1986) Nature 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) Science 240:1468-1474.

#### 4.11. Cell-free assays

Cell-free assays can be used to identify compounds which are capable of interacting with an F-box protein protein or binding partner, to thereby modify the activity of the F-box protein protein or binding partner. Such a compound can, e.g., modify the structure of an F-box protein protein or binding partner and thereby affect its activity. Cell-free assays can also be used to identify compounds which modulate the interaction between an F-box protein protein and an F-box protein binding partner, such as a target peptide. In a preferred embodiment, cell-free assays for identifying such compounds consist essentially in a reaction mixture containing

an F-box protein protein and a test compound or a library of test compounds in the presence or absence of a binding partner. A test compound can be, e.g., a derivative of an F-box protein binding partner, e.g., a biologically inactive target peptide, or a small molecule.

Accordingly, one exemplary screening assay of the present invention includes the steps of contacting an F-box protein protein or functional fragment thereof or an F-box protein binding partner with a test compound or library of test compounds and detecting the formation of complexes. For detection purposes, the molecule can be labeled with a specific marker and the test compound or library of test compounds labeled with a different marker. Interaction of a test compound with an F-box protein protein or fragment thereof or F-box protein binding partner can then be detected by determining the level of the two labels after an incubation step and a washing step. The presence of two labels after the washing step is indicative of an interaction.

An interaction between molecules can also be identified by using real-time BIA (Biomolecular Interaction Analysis, Pharmacia Biosensor AB) which detects surface plasmon resonance (SPR), an optical phenomenon. Detection depends on changes in the mass concentration of macromolecules at the biospecific interface, and does not require any labeling of interactants. In one embodiment, a library of test compounds can be immobilized on a sensor surface, e.g., which forms one wall of a micro-flow cell. A solution containing the F-box protein protein, functional fragment thereof, F-box protein analog or F-box protein binding partner is then flown continuously over the sensor surface. A change in the resonance angle as shown on a signal recording, indicates that an interaction has occurred. This technique is further described, e.g., in BIA technology Handbook by Pharmacia.

Another exemplary screening assay of the present invention includes the steps of (a) forming a reaction mixture including: (i) an F-box protein polypeptide, (ii) an F-box protein binding partner, and (iii) a test compound; and (b) detecting interaction of the F-box protein and the F-box protein binding protein. The F-box protein polypeptide and F-box protein binding partner can be produced recombinantly, purified from a source, e.g., plasma, or chemically synthesized, as described herein. A statistically significant change (potentiation or inhibition) in the interaction of the F-box protein and F-box protein binding protein in the presence of the test compound, relative to the interaction in the absence of the test compound, indicates a potential agonist (mimetic or potentiator) or antagonist (inhibitor) of F-box protein self-excision bioactivity for the test compound. The compounds of this assay can be contacted simultaneously. Alternatively, an F-box protein protein can first be contacted with a test

compound for an appropriate amount of time, following which the F-box protein binding partner is added to the reaction mixture. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified F-box protein polypeptide or binding partner is added to a composition containing the F-box protein binding partner or F-box protein polypeptide, and the formation of a complex is quantitated in the absence of the test compound.

Complex formation between an F-box protein protein and an F-box protein binding partner may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled F-box protein proteins or F-box protein binding partners, by immunoassay, or by chromatographic detection.

Typically, it will be desirable to immobilize either the F-box protein or its binding partner to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of an F-box protein to an F-box protein binding partner, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/F-box protein (GST/F-box protein) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the F-box protein binding partner, e.g. an <sup>35</sup>S-labeled F-box protein binding partner, and the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of F-box protein protein or F-box protein binding partner found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, either the F-box protein or its cognate binding partner can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated F-box



protein molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with an F-box protein can be derivatized to the wells of the plate, and F-box protein trapped in the wells by antibody conjugation. As above, preparations of an F-box protein binding protein and a test compound are incubated in the F-box protein presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the F-box protein binding partner, or which are reactive with F-box protein protein and compete with the binding partner; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the binding partner, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the F-box protein binding partner. To illustrate, the F-box protein binding partner can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diaminobenzadine tetrahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J Biol Chem 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as anti-F-box protein antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the F-box protein sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) J Biol Chem 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Cell-free assays can also be used to identify compounds which interact with an F-box protein protein and modulate an activity of an F-box protein protein. Accordingly, in one embodiment, an F-box protein protein is contacted with a test compound and the catalytic

activity of F-box protein is monitored. In one embodiment, the ability of the F-box protein to bind a target molecule is determined. The binding affinity of the F-box protein to a target molecule can be determined according to methods known in the art.

#### 4.12. Cell based assays

The invention further provides certain cell-based assays for the identification of F-box protein modulating agents which agonize or antagonize the self-excision activity of a wild type or conditional mutant F-box protein. In one embodiment, the effect of a test compound on the expression of an F-box protein-containing gene is determined by transfection experiments using a reporter gene comprising a conveniently assayed marker into which has been inserted the subject F-box protein polypeptide sequence. The reporter gene can be any gene encoding a protein which is readily quantifiable, e.g, the luciferase or CAT gene. Such reporter gene are well known in the art. The test compound is contacted with the reporter gene expressing cell line and the amount of reporter (e.g. CAT) activity produced in the presence of a test compound is compared to the amount of activity produced in the absence of the test compound.

In preferred embodiments, the cell-based assays of the present invention make use of the genetic complementation of a particular biological phenotype by the target F-box protein polypeptide for the purpose of identifying F-box protein self-excision agonist and antagonist compounds. For example, the complementation of a yeast *gal4* mutant phenotype, characterized by an inability to grow on a media containing galactose as the sole carbon source, by a GAL4:F-box protein hybrid protein is dependent upon F-box protein self-excision from the hybrid protein. Screening for F-box protein self-excision agonist and antagonist compounds may thus be effected by contacting the *gal4* GAL4:F-box protein yeast strain with a test compound and measuring a galactose growth characteristic in the presence and in the absence of the compound. Suitable galactose growth characteristics include colony size and doubling time on galactose media. An F-box protein self-excision may be used to identify agonist and antagonists which affect this galactose growth phenotype.

Another generally-applicable cell based assays useful for the identification of F-box protein self-excision agonists and antagonists is the yeast two-hybrid assay (Gyuris et al. (1993) Cell 75: 791-803) which is readily adaptable to isolating natural (e.g from a cDNA expression library) or synthetic (detected from a library of random open reading frames) polypeptides which interact with an F-box protein polypeptide of the invention. This F-box protein polypeptide/F-box protein polypeptide binding partner interaction can be further adapted

to screens which increase or decrease this F-box protein polypeptide/F-box protein polypeptide binding partner interaction, thereby allowing detection of F-box protein self-excision agonists and antagonists.

In a preferred embodiment of the invention, the chimeric polypeptide is introduced into a target cell by introducing and expressing in the target cell a nucleic acid encoding the chimeric polypeptide. In another embodiment of the invention, the chimeric polypeptide, or peptidomimetic thereof is introduced directly into the target cell. The method of administration of a chimeric polypeptide to a target cell may depend on various factors, including but not limited to whether the target cell is in a subject or ex vivo, whether prolonged or temporally limited presence of the chimeric polypeptide is desired and which method of administration may be more effective for a particular type of cell.

The target cell can be any cell which contains the necessary components of the ubiquitin proteolytic pathway and a target polypeptide. In a preferred embodiment, the cell is a eukaryotic cell, preferably a mammalian cell and even more preferably a human cell. However, the target cell can also be a murine, rat, canine, feline, bovine, ovine, porcine, goat, equine, or a primate cell. A target cell can also be a yeast cell, e.g., *Saccharomyces Cerevisiae* cell.

The target cell can be any type of cell, present in a subject or outside of a subject. For example, the cell in which a target polypeptide is to be degraded can be in a subject and the chimeric polypeptide or nucleic acid encoding such is administered to the subject. Alternatively, the target cell can be obtained from a subject, the chimeric polypeptide or nucleic acid encoding such is introduced into the target cell and the target cell is optionally administered to the same or another subject. In yet another embodiment, the target cell is not obtained directly from a subject, but is a cell from a cell culture and the chimeric polypeptide or nucleic acid encoding such is introduced in vitro into the target cell. Accordingly, the target cell can be a cell from any established cell line, which can be obtained, e.g., from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD).

The target cell can be a cell in suspension or can be part of a tissue or organ. The cell can be an undifferentiated cell, e.g., a blast cell, or the cell can be a differentiated or partially differentiated cell. The cell can be a somatic cell or a germ cell. The cell can be a normal cell or a cell from a tumor, e.g., malignant or benign tumor. The cell can be a blood cell, such as a lymphocyte, a granulocyte, an eosinophil, a basophil, an erythrocyte. In other embodiments, the cell can be a muscle cell, a renal cell, a liver cell, an epithelial cell, a bone cell (e.g., osteoblast or osteocyte), a cartilage cell, mesenchymal cell, endothelial cell, brain cell, or any other cell type,

so long as the cell contains the necessary elements of the ubiquitin proteolysis pathway, or can be modified to contain these components.

A nucleic acid encoding a chimeric polypeptide of the invention is preferably included in a vector, e.g., a eukaryotic expression vector. The vector preferably comprises a promoter and may contain enhancer and other regulatory elements. The promoter can be cell-type specific, or the promoter can be expressible in different types of cells. The promoter can also be constitutive. Alternatively, the promoter can be inducible. An inducible promoter is useful in embodiments in which the target polypeptide is to be degraded only at certain times, in a controllable manner.

Ligating nucleic acid fragments into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial) cells, are standard procedures well known in the art, and described in references cited herein.

Vectors that allow expression of a nucleic acid in a cell are referred to as expression vectors. Typically, expression vectors used for expressing a chimeric polypeptide contain a nucleic acid encoding a chimeric polypeptide, operably linked to at least one transcriptional regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject chimeric proteins. Transcriptional regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990)..

Suitable vectors for the expression of a chimeric polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 *ori*, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic

cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

A vector for use in the invention may also contain additional regulatory elements, such as elements which regulate the stability of the mRNA encoding the chimeric polypeptide or elements which regulate the level of transduction of the mRNA or the stability of the chimeric polypeptide. Such elements are well known in the art.

The invention also provides nucleic acids, and vectors comprising such, which encode an E3 recruiting domain. In a preferred embodiment, the nucleic acid further comprises the nucleotide sequence of at least one restriction enzyme recognition site, which allows in frame insertion of a nucleic acid encoding a target protein binding domain, such that expression of the nucleic acid produces a chimeric polypeptide. The restriction enzyme site can be located upstream or downstream of the nucleotide sequence encoding the E3 recruiting domain.

Vectors encoding chimeric polypeptides can be introduced into target cells by various methods. The choice of the method may depend on the type of target cell and whether the vector is to be introduced into cells in a host, i.e., in vivo, or ex vivo.

In certain embodiments, it may be preferable to directly introduce a chimeric polypeptide or peptidomimetic thereof into a target cell, as opposed to inserting a nucleic acid encoding such.

The polypeptides can be synthesized in vitro or alternatively, the polypeptides of the invention can be produced in a cell, isolated and introduced into the target cell. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. Suitable media for cell culture are well known in the art. The recombinant polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant polypeptide

is a fusion protein containing a domain which facilitates its purification, such as GST fusion protein or a Histidine tagged protein.

In some instances, it may be desirable to express the recombinant ACE-2 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

Methods for introducing polypeptides or peptidomimetics into cells are well known in the art and include, e.g., use of liposomes.

The methods and compositions of the invention provide a means for degrading any desired protein in a cell, so long as the target polypeptide can be recruited to an E3 complex and thereby be degraded. Accordingly, the invention provides methods and compositions for treating or preventing any disease or condition in a subject, which is caused by or contributed to by the presence of an abnormal amount of a specific polypeptide. The invention can be used to reduce the amount of a target protein to various extends as well as to eliminate essentially all of the target polypeptide from the target cell.

The target polypeptide can be a cytoplasmic polypeptide, a nuclear polypeptide or a membranous polypeptide, e.g., a polypeptide attached to the cytoplasmic membrane.

In one embodiment, the target polypeptide is involved in regulating the growth and/or differentiation and/or death of a cell. For example, the target polypeptide can be an oncoprotein, the presence of which contributes to the immortalization of a cell. Accordingly, the invention provides methods and compositions for treating cancer and other proliferative disorders.

In another embodiment, the target polypeptide is that of a microorganism, e.g., a virus or intracellular bacteria. Preferred polypeptides are those which are necessary for the survival and/or reproduction of the microorganism. Thus, methods involving degradation of such polypeptides can be used for treating and/or preventing infections by microorganisms.

Also within the scope of the invention are methods for specifically eliminating cells in a subject, by targeting and degrading polypeptides which are essential for the survival of the target cell. Thus, controlling expression of the chimeric polypeptide with an inducible promoter provides a method for controlled cell death. Such a method is particularly useful in gene therapy methods, by providing a safety mechanism, by which genetically engineered can

be destroyed upon administration of a compound inducing expression of the chimeric polypeptide.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. As used herein "pharmaceutical compositions" include any compositions that may be administered to treat a subject according to the method of the invention, and may comprise a chimeric polypeptide, peptidomimetic, nucleic acid encoding a chimeric polypeptide and/or other desirable compounds. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by, for example, injection, inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For such therapy, the pharmaceutical compositions of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the pharmaceutical compositions of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the pharmaceutical compositions may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., ationd oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form

of tablets or lozenges formulated in conventional manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the pharmaceutical compositions may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the pharmaceutical compositions of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. A wash solution can be used locally to treat an injury or inflammation to accelerate healing.

In clinical settings, a gene delivery system of the chimeric polypeptide encoding nucleic acid can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g., by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene



delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g., Chen et al. (1994) *PNAS* 91: 3054-3057). A nucleic acid encoding a chimeric polypeptide can be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct or compound of the invention can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle or compound is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The invention provides a quick, easy, and economic alternative to the gene knock-out technology to eliminate any cellular protein in tissue culture or in animals. For example, a chimeric polypeptide or nucleic acid encoding such can be introduced into embryonic stem cells or in a blastocyst (for preparing transgenic animals), but also at any later stage of development of the embryo or animal, or even in an adult animal. Thus, the target polypeptide can be degraded from the early developmental stages on, or it can be degraded only at specific developmental stages or in a mature animal. This allows the study of the function of a specific target polypeptide, by specifically eliminating the polypeptide in the desired cell or in all the cells of an animal.

The method of the invention will be useful, e.g., in drug discovery. For example, specific degradation of a protein of interest can be used to determine the effect of a drug that would eliminate or inhibit the activity of the protein of interest, and thus whether this protein is an ideal target for drug intervention.

In another embodiment, the invention provides a screening method for identifying compounds which modulate, i.e., inhibit or stimulate, the interaction between two molecules. In a preferred embodiment, the invention provides a method for identifying compounds which inhibit the binding of two polypeptides to each other. For example, a method for identifying a

compound which inhibits the interaction between polypeptide X and polypeptide Y comprises expressing in a cell a chimeric polypeptide comprising (i) an E3 recruiting domain, e.g., an F-box and WD repeats of the human h- $\beta$ TrCP protein and (ii) a portion of polypeptide X, sufficient for binding to polypeptide Y. Optionally, a nucleic acid encoding polypeptide Y, or a portion thereof sufficient for binding to polypeptide X, can also be introduced and expressed in the cell. Polypeptide Y (the target polypeptide) can also be linked to a marker peptide which can easily be detected, e.g., by using an antibody to the marker peptide. For example, a marker peptide can be a myc tag or a peptide of the *Influenza* virus, for which highly specific antibodies are commercially available. The cell can then be contacted with a test compound and the presence and/or amount of polypeptide Y is determined. Any of a variety of techniques for detecting the presence and/or amount of polypeptide Y can be used. For example, methods including the use of an antibody directed to polypeptide Y or to a marker peptide linked to polypeptide Y can be used, and include methods such as immunoprecipitations and Western Blots, immunohistochemistry. Alternatively, polypeptide Y can be labeled, e.g., radioactively labeled, and the presence and/or amount of polypeptide Y is determined, e.g., by measuring the amount of radioactivity that is precipitated with an antibody that binds specifically to polypeptide Y. The presence of a higher level of polypeptide Y in a cell contacted with a test compound relative to the level of polypeptide Y present in an identical cell which was not contacted with the test compound indicates that the test compound inhibits the interaction between polypeptide X and polypeptide Y.

In another embodiment of the above-described screening assay, the polypeptide comprising a portion of polypeptide Y sufficient for binding to X, further comprises a cell death inducing domain, e.g., from the Fas or Apo-1 protein, such that the presence of a sufficient amount of Y in a cell induces the cell to undergo cell death. In this case, contacting the cell with a compound which inhibits the interaction between the polypeptides X and Y will result in cell death, whereas contacting the cell with a compound which does not inhibit binding of the two polypeptides to each other will not result in cell death since the death inducing polypeptide will be degraded in the cell by the ubiquitin proteolytic pathway. In this embodiment it may be preferable to control the presence of Y in a cell by, e.g., operably linking the nucleic acid encoding Y to an inducible promoter.

Thus, the method of the invention provides an easy and reliable screening assay for identifying compounds which inhibit or stimulate the interaction between two polypeptides in a cell, in particular a mammalian cell.

The method of the invention also provides a powerful technology for the functional genomic studies to analyze the functions of novel genes identified by the human genome project.

Furthermore, the function of proteins interacting with a specific protein of interest can be determined by specifically targeting the degradation of one or more of these interacting proteins. In addition, through systematic elimination of multiple interacting proteins and comparison of the phenotypes generated from each targeted proteolysis, an overall picture of the entire pathway of these proteins can be obtained. Thus, in addition to the numerous therapeutic uses for the method of the invention, the method is a useful experimental tool that can be used for elucidating the biological activity of any protein of interest. The information derived from this research can then be used for diagnostic, prognostic and therapeutic purposes.

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

## 5. Examples

*Cdc4p is an unstable component of the SCF<sup>Cdc4p</sup> complex*

The Cdc34p/SCF<sup>Cdc4p</sup> pathway appears to operate at cell cycle stages other than the G1/S transition (King et al. (1996) Science 274: 1652-9), suggesting that the components involved in the Cdc34p/SCF<sup>Cdc4p</sup> pathway must be physically present throughout the cell cycle. Indeed, the steady-state level of Cdc34p does not fluctuate during the cell cycle (Goebl et al. (1994) Mol Cell Biol 14: 3022-9). To test whether the components comprising the SCF<sup>Cdc4p</sup> complex are stable and available throughout the cell cycle, we examined the half life of individual SCF<sup>Cdc4p</sup> components in exponentially growing yeast cells. Expression of epitope-tagged Cdc4p, Cdc53p and Skp1p under the control of the galactose-inducible *GALI-10* promoter was induced for 1 hour in exponentially growing KY130 cells, and the stability of the proteins was determined following promoter shut-off by switching cells into glucose media containing cyclohexamide (Visintin et al. (1997) Science 278: 460-3). As shown in Fig. 1A, Cdc53p and Skp1p were stable ( $T_{1/2} > 60'$ ), whereas Cdc4p was short-lived, with a half life of 15 minutes, similar to that of Sic1p (Verma et al. (1997) Science 278: 455-60). To examine whether the observed instability of Cdc4p was an intrinsic property and not due to heterologous expression of epitop tagged Cdc4p, we examined the half life of endogenous Cdc4p, which is expressed at very low levels in wild type cells. By pulse chase, we found that endogenous Cdc4p was also unstable, with a  $t_{1/2}$  comparable to what was observed for the transfected Cdc4p (Fig. 1B). This experiment also independently confirmed the stability of the endogenous Skp1p (Fig. 1B). Since Cdc34p/SCF<sup>Cdc4p</sup> proteolytic activity has been implicated in multiple cell cycle stages (Bai et al. (1996) Cell 86: 263-74; Schwob et al. (1994) Cell 79: 233-44), we next analyzed the half life of Cdc4p in cells synchronized at specific stages of the cell cycle. As shown in Fig. 1C, Cdc4p was rapidly degraded in synchronized cells upon release from alpha-factor mediated arrest in G1 phase, from hydroxyurea arrest in S phase, or from nocodazole arrest in M phase, demonstrating that Cdc4p is degraded throughout the cell cycle. These data indicated that Cdc4p was the only short-lived component of the Cdc34p/SCF<sup>Cdc4p</sup> complex, and that intact SCF<sup>Cdc4p</sup> existed as a transient complex *in vivo*.

**Figure 1** shows that Cdc4p is the only unstable component of the SCF<sup>Cdc4p</sup> ubiquitin protein ligase complex. (A) Exponentially growing KY130 cells carrying either Flag-Cdc4p, HA-Cdc53p, Flag-Skp1p or Sic1p-HA under the control of the *GALI-10* promoter, were induced to express the individually tagged proteins for 1 hour by 2% galactose, and their half-lives were determined by promoter shut-off and immunoblotting using anti-Flag (M2) or HA.11 monoclonal antibodies to detect F-Cdc4p, Sic1p-HA, HA-Cdc53p and F-Skp1p, respectively.

As a loading control, endogenous Cdc28p was visualized by immunoblotting using an anti-Cdc28p antibody. "o" is an unknown species in yeast extracts which cross-reacts with the anti-Flag M2 monoclonal antibody. (B) The endogenous Cdc4p is short-lived. Pulse-chase analysis was carried out in exponentially growing KY130 cells, and the decay of the endogenous Cdc4p or Sklp1p over time were determined by immunoprecipitation with an affinity purified anti-Cdc4p polyclonal antibody or an anti-Sklp1p antibody. <sup>35</sup>S-labeled Cdc4p or Sklp1p immunoprecipitates were subjected to SDS-PAGE and were visualized after 4 days of exposure. (C) KY130 cells carrying GAL-F-Cdc4p were first arrested by ( $\alpha$ -factor in G1 phase, hydroxyurea in S phase or nocodazole in M phase (Visintin et al., 1997). Following the release from growth arrest, the levels of F-Cdc4p over the indicated time periods upon promoter shut-off was determined by immunoprecipitation and visualized by immunoblotting using the anti-Flag M2 antibody. The M2 cross-reacting species in (A) was not present in the immunoprecipitates.

#### Cdc4p is Degraded by the Ubiquitin-Proteasome Pathway

We next examined whether Cdc4p proteolysis was ubiquitin dependent by testing directly whether Cdc4p was ubiquitinated *in vivo*. Since endogenous Cdc4p is present at very low levels and because multiubiquitinated species are quickly degraded by the 26S proteasome; high copy number plasmids expressing Cdc4p either alone or together with Myc-tagged ubiquitin were introduced into wild type yeast cells to facilitate detection of ubiquitin-conjugated Cdc4p intermediates. Myc-tagged ubiquitin can be efficiently conjugated to proteins to form multi-ubiquitinated chains (Ellison and Hochstrasser (1991) J Biol Chem 266: 21150-7). Furthermore, addition of the Myc-epitope on ubiquitin results in a distinct electrophoretic pattern of slower migrating species on SDS PAGE when compared to that of the protein conjugated to native ubiquitin, thus serving as a specific indicator of ubiquitinated intermediates (Ellison and Hochstrasser (1991) J Biol Chem 266: 21150-7). Cdc4p was immunoprecipitated from extracts of exponentially growing cells using an anti-Cdc4p polyclonal antibody, blotted and probed with the 9E10 monoclonal antibody for Myc-Ub, and with the Cdc4p-specific antibody. In Cdc4p and Myc-Ub expressing cells, a ladder of slower migrating species was detected by the Cdc4p specific antibody as well as the Myc antibody that recognizes Myc-Ub conjugated proteins (Fig. 2A, lanes 4 and 8). In cells expressing Cdc4p alone, a similar spectrum of bands was observed with the Cdc4p specific antibody, but not the Myc antibody (Fig. 2A, lanes 3 and 7). However, slower migrating species of Cdc4p could be detected using the anti-ubiquitin antibody (data not shown), identifying the slow migrating species in Cdc4p expressing cells (Fig. 2A, lane 3) as

ubiquitinated conjugates. Furthermore, Myc-ubiquitin conjugates of endogenous Cdc4p could be observed upon longer exposure of lane 6 (Fig. 2A, lanes 10). The Cdc4p-Myc-Ub<sub>n</sub> species had slower electrophoretic mobility compared to the Cdc4p-Ub<sub>n</sub> species due to the addition of the Myc-tag on the ubiquitin (Fig. 2A, compare lanes 3 and 4). These data provide unambiguous evidence that the slow migrating species of Cdc4p are ubiquitinated. Furthermore, Cdc4p degradation was inhibited in yeast cells carrying the temperature sensitive *prel-1*, *pre4-1* double mutations of the 20S proteasome subunits at 37°C (Fig. 2B) (Hilt et al. (1993) J Biol Chem 268: 3479-86), indicating that Cdc4p is subjected to proteasome mediated proteolysis. Taken together with the rapid turnover rate of Cdc4p, these experiments indicate that the F-box-containing component of SCF<sup>Cdc4p</sup> is itself subject to degradation by the ubiquitin-proteasome pathway.

**Figure 2** shows that Cdc4p is ubiquitinated and degraded by the proteasome *in vivo*. (A) INVSc1 cells expressing Myc-Ub alone (lanes 2 and 6), or Cdc4p alone (lanes 3 and 7), or Myc-Ub and Cdc4p (lanes 4 and 8) were immunoprecipitated with the anti-Cdc4p polyclonal antibody, and were immunoblotted with either anti-Cdc4p antibody or the 9E10 anti-Myc antibody which recognized Myc-tagged ubiquitin. INVSc1 cells carrying expression vectors alone (lanes 1, 5 and 9) were used as a negative control. The arrowhead indicates the endogenous Cdc4p (lanes 1 and 2) or ectopically expressed Cdc4p (lanes 3 and 4). Upon 5X prolonged exposure, the endogenous Cdc4p was shown to be modified by the Myc-Ub (lane 10). "." and "\*" represents Cdc4p modified by endogenous ubiquitin or Myc-Ub, respectively. "o" indicates a cellular protein cross-reactive with the Cdc4p antibody. (B) The stability of the F-Cdc4p in YY371 and YY374 cells was assessed by promoter shut-off at 37°C. "o" is an unknown protein cross-reacting with the anti-Flag M2 monoclonal antibody.

*The F-box-containing Grrlp of the SCF<sup>Grrlp</sup> is also Ubiquitinated and Rapidly Degraded.*

To investigate whether the rapid turnover of Cdc4p is a specific feature of that protein, or whether it is a characteristic shared by other F-box-containing ubiquitin protein ligases, we examined the stability of the F-box-containing Grrlp of SCF<sup>Grrlp</sup> (Barral et al. (1995) Genes Dev 9: 399-409; Li and Johnston (1997) EMBO J 16: 5629-38; Skowyra et al. (1997) Cell 91: 209-19). HA-tagged Grrlp, lacking the N-terminal 280 amino acids (HA-Grrlp<sup>ΔN</sup>), has previously been shown to be fully functional since it can complement a *grr1* null allele (Li and Johnston (1997) EMBO J 5629-38). The half life of HA-Grrlp<sup>ΔN</sup> was determined in wild type cells constitutively expressing HA-Grrlp<sup>ΔN</sup>. In a pulse chase analysis shown in Fig. 5A, HA-Grrlp<sup>ΔN</sup> was rapidly degraded, with a t<sub>1/2</sub> of 30 minutes. Ubiquitin conjugates of HA-Grrlp<sup>ΔN</sup>

were demonstrated by the marked enhancement of a HA-Grrlp<sup>ΔN</sup> ubiquitin ladder when exogenous ubiquitin was also overexpressed (Fig. 5B, compare lanes 3 and 4). Furthermore, the spectrum of the HA-Grrlp<sup>ΔN</sup>-Myc-Ub species exhibited the characteristic shift of the electrophoretic mobility compared to ubiquitinated HA-Grrlp<sup>ΔN</sup> proteins conjugated with the native ubiquitin (Fig. 5B, lanes 3, 4 and 5) (Ellison and Hochstrasser (1991) J Biol Chem 266: 21150-7). In Myc-Ub expressing cells, the slower migrating HA-Grrlp<sup>ΔN</sup> conjugates were immunoreactive with anti-Myc antibody (data not shown). These results indicate that HA-Grrlp<sup>ΔN</sup> is indeed ubiquitinated inside the cell. An analogous SCF<sup>Skp2</sup> complex was recently described in human cells (Lisztwan et al. (1998) EMBO J 17: 368-83). Interestingly, the protein level of the human homolog of Grrlp, p45<sup>Skp2</sup>, decreases during the M phase of the cell cycle. Although the abundance of the *SKP2* mRNA is cell cycle regulated (Zhang et al. (1995) Cell 81: 1085-95), ubiquitin-dependent proteolysis may also play a role since addition of the proteasome inhibitor LLnL prevents the decrease of Skp2 protein levels in M phase (Lisztwan et al. (1998) EMBO J 17: 368-83). Therefore, we propose that ubiquitin-dependent proteolysis of F-box-containing substrate recognition component in SCFs is likely a general scheme for the function of the SCF ubiquitin-protein ligases.

**Figure 5** shows that Grrlp is also a short-lived protein and is ubiquitinated *in vivo*. (A) Wild type Y81 cells constitutively expressing HA-GRR1<sup>ΔN</sup> were grown to mid-log phase, and the half-life of HA-Grrlp<sup>ΔN</sup> was determined by pulse-chase analysis and immunoprecipitation with the HA.11 anti-HA polyclonal antibody (Babco) as in Fig. 1B. (B) Y81 cells expressing Myc-Ub alone (lanes 2), or HA-Grrlp<sup>ΔN</sup> alone (lanes 3), Ub and HA-Grrlp<sup>ΔN</sup> (lanes 4 or Myc-Ub and HA-Grrlp<sup>ΔN</sup> (lanes 5) were immunoprecipitated with the HA.11 anti-HA polyclonal antibody (Babco) and immunoblotted with the HA.11 monoclonal antibody (Babco) for HA-Grrlp<sup>ΔN</sup>. “.” and “\*” are examples of HA-Grrlp<sup>ΔN</sup> conjugated to ubiquitin or Myc-Ub, respectively. The slower migrating HA-Grrlp<sup>ΔN</sup> conjugates marked by “\*” were also detected by anti-Myc monoclonal antibody (not shown). “o” indicates unknown species in Y81 extracts that cross-react with HA.11 monoclonal antibody.

#### Cdc4p Proteolysis is Mediated by the Cdc34p/SCF<sup>Cdc4p</sup> Machinery

The fact that one of the SCF components is also degraded by the ubiquitin-proteasome pathway raised the question of whether the Cdc34p/SCF<sup>Cdc4p</sup> machinery that degrades phosphorylated substrates is the same machinery that promotes ubiquitination of Cdc4p, or whether a separate pathway exists for ubiquitin dependent proteolysis of Cdc4p. We therefore

analyzed Cdc4p stability in *cdc34-2* and *skpl-11* temperature sensitive cells. It has been shown that the Cdc4p substrate, Sic1p, is stable in *cdc34-2* and *skpl-11* cells at the non-permissive temperature due to the inactivation of the Cdc34p ubiquitin-conjugating enzyme and the Skplp component of SCF<sup>Cdc4p</sup>, respectively (Bai et al. (1996) Cell 86: 263-74; Schwob et al. (1994) Cell 79: 233-44). In both *cdc34-2* and *skpl-11* mutants, Cdc4p accumulated to a high steady state level, and its degradation was dramatically inhibited at the non-permissive temperature with t1/2 >60 minutes (Fig. 3). As a control, we showed that when the temperature was raised from 25°C to 37°C, F-Cdc4p was degraded at a slightly higher rate in the respective isogenic wild type cells (data not shown). Since Cdc28p dependent phosphorylation is a prerequisite for Sic1p recognition by Cdc4p and its subsequent ubiquitination by the Cdc34p/SCF<sup>Cdc4p</sup> pathway (Feldman et al. (1997) Cell 91: 221-30; Schneider et al. (1996); Schwob et al. (1994) Cell 79: 233-44; Skowyra et al. (1997) Cell 91: 209-19; Verma et al. (1997) Science 278: 455-60), we next examined whether the Cdc28p kinase also plays a role in the degradation of Cdc4p itself. In *cdc28-4* temperature sensitive cells, Sic1p degradation is impaired at the non-permissive temperature (37°C) and the cells are arrested at the G1/S boundary (Schwob et al. (1994)). However, Cdc4p was still rapidly degraded at 37°C (Fig. 3), indicating that the inhibition of Cdc4p turnover in *cdc34-2* and *skpl-11* was not due to a general cell cycle arrest, but rather resulted from the specific inactivation of Cdc34p and Skplp, respectively. Thus, we conclude that Cdc4p is auto-ubiquitinated by the Cdc34p/SCF<sup>Cdc4p</sup> machinery. Whereas Sic1p degradation is triggered by Cdc28p-dependent phosphorylation at a specific stage of cell cycle, our results indicate that Cdc4p proteolysis is constitutive and not subject to regulation by the Cdc28p kinase (Fig. 1C, Fig. 3).

**Figure 3** shows that Cdc4p is stabilized in *cdc34-2* and *skpl-11*, but not *cdc28-4* temperature sensitive mutant cells. The half-life of the galactose inducible F-Cdc4p in *cdc34-2* (KY203), *skpl-11* (Y553) or *cdc28-4* cells was determined by promoter shut-off at the non-restrictive (25°C) or restrictive temperatures (37°C). The decay of the F-Cdc4p protein over time was measured by immunoblotting (*skpl-11* and *cdc28-4*) or immunoprecipitation followed by immunoblotting (*cdc34-2*) using the anti-Flag antibody. "o" is an unknown species cross-reacting with the anti-Flag M2 monoclonal antibody.

#### Functional Association of Cdc4p with the Core SCF is Necessary for its Destruction

The Cdc4p F-box has been shown to directly interact with Skp1p in the assembly of the SCF<sup>Cdc4p</sup> complex (Bai et al. (1996) Cell 86: 263-74; Skowyra et al. (1997) Cell 91: 209-



19). Since efficient transfer of ubiquitin to Sic1p requires the direct interaction of Sic1p with the Cdc34p/SCF<sup>Cdc4p</sup> machinery, mutant Cdc4p defective in its binding to Skp1p should be resistant to degradation. To test this hypothesis, we engineered two mutant forms of Cdc4p, containing either a Gly 300 to Asp substitution in the F-box corresponding to the *cdc4-5<sup>s</sup>* allele (Cdc4p(G300D)), or a deletion of the entire F-Box (Cdc4p( $\Delta$ F)) (Bai et al. (1996) Cell 86: 263-74). The binding of these mutant proteins to Skp1p was reduced 10 to 15 fold compared to wild type Cdc4p protein in the *in vitro* binding assays (data not shown). These two mutant Cdc4p proteins were transiently expressed in KY130 cells under the control of the *GAL1-10* promoter at 30°C, and their half lives-were examined after promoter shut-off. As shown in Fig. 4A, the Cdc4p(G300D) and Cdc4p( $\Delta$ F) proteins were both stable, with half lives greater than 60 minutes. In addition, cells expressing the stable Cdc4p(G300D) mutant exhibited severe growth defects. The *cdc4-5* allele bearing the G300D mutation at the chromosomal *CDC4* locus (Bai et al., 1996), could only grow at the low permissive temperature (23°C), and was completely arrested in G1 at 34°C with large and multiply budded cells (Fig. 4B). This is likely due to the inability of the Cdc4p(G300D) mutant to target its substrates to the SCF machinery for destruction. A similar partial phenotype was observed in KY130 cells ectopically expressing Cdc4p( $\Delta$ F). These cells displayed a 2- to 3-fold reduced growth rate compared to cells expressing wild type Cdc4p. Microscopic examination revealed an increased population of large, multiply budded cells, morphologically similar to the G1 arrested *cdc4<sup>s</sup>* cells grown at the non-permissive temperature (Johnson (1991) Thesis in Genetics, Univ. of WA, Seattle; Schwob et al., (1994) Cell 79: 233-44) (Fig. 4B). However, complete growth arrest was not observed since high level expression of the Cdc4p( $\Delta$ F) mutant protein could not be sustained in actively dividing KY130 cells for reasons yet to be determined (data not shown). In any case, these observations indicate that binding of Cdc4p with the core SCF is necessary for its proteolysis, and that non-degradable Cdc4p mutant proteins may negatively affect cell growth.

**Figure 4**, panels **A** and **B** show that the F-box and WD40 domains of Cdc4p target Cdc4p for rapid proteolysis, and that Cdc4p function is required for cell cycle progression from G1 to S. (A) Flag-tagged Cdc4p mutants carrying either a Gly300 to Asp substitution, or an internal deletion of the entire F-box were induced to express in KY130 cells for 1 hour. The half life of the mutant Cdc4p proteins were determined by promoter shut-off as in Fig. 1A. "o" represents the M2 cross-reacting species. (B) Morphology of SJ1012-4 (*cdc4-5*) cells in YEPD medium at 23°C (left) or kept at 34°C for 6 hours (right).

Targeted Degradation of a Polypeptide by Fusion to Cdc4p F-box and WD40 repeats

Since Cdc4p is targeted for auto-ubiquitination within the SCF<sup>Cdc4p</sup> complex, we predicted that any proteins, either covalently linked or in association with Cdc4p, would also be targeted within SCF<sup>Cdc4p</sup> for rapid degradation. As an initial test of this hypothesis, a Cdc4p segment containing the F-box and the WD40 repeats (Cdc4p<sup>FWD</sup>) was fused to the stable  $\beta$ -galactosidase protein (t<sub>1/2</sub> >20 hr) (Bachmair et al. (1986) Science 234: 179-86), and introduced into KY130 cells under the control of the *GAL1-10* promoter. This fusion protein is functional since it can complement the temperature sensitivity of a *cdc4<sup>ts</sup>* allele (Johnson (1991) Thesis in Genetics, Univ. of WA, Seattle). As shown in Fig. 4C, the Cdc4p<sup>FWD</sup>- $\beta$ gal fusion was indeed highly unstable, being degraded with a half life of 35 minutes. However, the same Cdc4p<sup>FWD</sup>- $\beta$ gal fusion protein containing the Gly 300  $\rightarrow$  Asp F-box mutation was stable, further suggesting that Cdc4p dependent proteolysis requires a functional interaction with Skp1p (Fig. 4C). Furthermore, Cdc4p segments that lacked either the F-Box or the WD40 repeats (Cdc4p<sup>WD</sup> or Cdc4p<sup>F</sup>) failed to target degradation of the fused  $\beta$ -galactosidase (Fig. 4C), indicating that both the F-box and the WD40 domains are required for the SCF<sup>Cdc4p</sup> dependent degradation of the Cdc4p fusion proteins. Deletion of the last three WD repeats of Cdc4p has no effect on Cdc4p/Skp1p/Cdc53p complex formation (Skowyra et al. (1997) Cell 91: 209-19). However, this truncated Cdc4p fails to interact with phosphorylated Sic1p (Skowyra et al. (1997) Cell 91: 209-19). Collectively, these data suggest that the F-box and the WD40 repeat components of Cdc4p are both required for the assembly of a macromolecular Cdc34p/SCF<sup>Cdc4p</sup>/substrate complex optimal for ubiquitin transfer to Cdc4p and its substrates.

The F-box of Cdc4p contains 42 amino acids and has been biochemically defined as the Skp1p interaction domain (Bai et al. (1996) Cell 86: 263-74). To further analyze the role of F-box for SCF functions, the F-box of Cdc4p was overexpressed in the wild type KY130 cells as a  $\beta$ -galactosidase fusion and cell growth was examined after 2 days. As shown in Fig. 4D, overexpression of the non-degradable Cdc4p<sup>FWD</sup>- $\beta$ gal strongly inhibited cell growth, whereas neither the short-lived Cdc4p<sup>FWD</sup>- $\beta$ gal, nor the stable Cdc4p<sup>WD</sup>- $\beta$ gal fusion proteins caused growth inhibition (Fig. 4D). We further showed that the Cdc4p<sup>F</sup>- $\beta$ gal could enter the core SCF as Skp1p and Cdc53p could be readily detected in the Cdc4p<sup>F</sup>- $\beta$ gal immunoprecipitates (P. Zhou and P. Howley, unpublished result). This suggested that the F-box alone, when present at high intracellular levels, might exert dominant negative effects through blocking the access of the core SCF by the F-box proteins, therefore interfering with SCF-mediated degradation of substrates or auto-ubiquitination of the F-box-containing SCF components. Interestingly, the entire coding

regions of the adenovirus E3-12.9K or the Baculovirus ORF11 are exclusively composed of F-box-like sequences (Bai et al. (1996) Cell 86: 263-74). Given that overexpression of the Cdc4p F-box alone inhibited cell growth, it would be important to investigate whether these viral proteins might similarly perturb cell cycle regulation through interfering with the SCF functions.

**Figure 4**, panels **C** and **D** show that the F-box and WD40 domains of Cdc4p target proteins connected to Cdc4p for rapid proteolysis. (C) The stable *E. coli*  $\beta$ -galactosidase, when fused to Cdc4p<sup>F/WD</sup>, was rapidly degraded. Segments of Cdc4p including (1) F-box and WD40 repeats (Cdc4p<sup>F/WD</sup>), "●", (2) Cdc4p<sup>F/WD</sup>(G300D) "Δ", (3) F-box (Cdc4p<sup>F</sup>), "■" or (4) WD40 repeats (Cdc4p<sup>WD</sup>) "◆" were fused to *E. coli*  $\beta$ gal and under the control of the *GAL1-10* promoter. The decay of each  $\beta$ gal fusion over time was assessed in KY130 cells by promoter shut-off and immunoblotting using an anti- $\beta$ gal monoclonal antibody, and was quantitated by densitometer scanning. Logarithmic values were assigned to densitometry-determined protein levels and the percentage of protein remaining was plotted against time (minutes). (D) Overexpression of the F-box as a  $\beta$ -galactosidase fusion inhibits cell growth. KY130 cells carrying Cdc4p<sup>F/WD</sup>- $\beta$ -Gal (F/WD), Cdc4p<sup>F</sup>- $\beta$ -Gal (F) or Cdc4p<sup>WD</sup>- $\beta$ -Gal (WD) expression plasmids were streaked on medium containing glucose (left) or galactose (right) and incubated at 30°C for 2 days.

#### Targeted Degradation of the Retinoblastoma Tumor Suppressor in Yeast

To demonstrate that a selected target polypeptide could be degraded by recruitment to an F-box-containing SCF complex, the retinoblastoma tumor suppressor protein (pRB) was expressed in yeast and targeted for degradation using F-box-pRB interaction domain fusion proteins. We chose the retinoblastoma protein pRB as a proteolysis target, since pRB was known to express at high steady state levels in yeast without interfering with normal growth (Hatakeyama et al., (1994) Genes Dev 8: 1759-71). In addition, the interactions between pRB and viral oncoproteins have been extensively characterized. The pRB expression plasmid p2202TRB under the control of the *GALI-10* promoter was introduced into wild type Y81 cells, and the half life of pRB was determined by promoter shut-off following transient induction of pRB by galactose. As shown in Fig. 7A, pRB was highly stable and no degradation was observed within 90 minutes upon promoter shut-off. This result also indicates that although cell cycle dependent phosphorylation of pRB in mammalian cells is faithfully mimicked in *S. cerevisiae* (Hatakeyama et al., (1994) Genes Dev 8: 1759-71), the Cdc34p/SCF<sup>Cdc4p</sup> machinery

that degrades phosphorylated Sic 1p during G1/S transition can not recognize either hyper- or hypo-phosphorylated pRB.

We next engineered two Cdc4p derivatives that were capable of interacting with pRB. These engineered F-box-containing hybrid proteins were composed of two functional entities: the F-box and WD repeat domains of Cdc4p (Cdc4p<sup>FWD</sup>) sufficient for binding to Sklp and targeting to the core SCF, and the pRB binding domains of either the simian virus 40 Large T antigen (residues 103-115), designated LTP (for Large T antigen Peptide), or the papillomavirus oncoprotein E7 (residues 2-35), designated E7N (Munger et al. (1989) EMBO J 8: 4099-105). As negative controls, the same domains of each viral oncoprotein carrying point mutations LTP(FGSK) or internal deletions E7N( $\Delta$ DLYC) within the conserved LXCXE pRB binding motif, thus abolishing their interactions with pRB, were also covalently attached to Cdc4p<sup>FWD</sup> (DeCaprio et al. (1989) Cell 58:1085-95; Dyson et al. (1989) Science 243: 934-7; Figge et al. (1993) Protein Sci 2: 155-64; Munger et al. (1989) EMBO J 8: 40999-105; Yang et al. (1995) Nucleic Acids Res 23: 1152-6). These engineered F-box proteins allowed us to address two questions: 1) whether a stable protein (pRB) can now be degraded by an engineered Cdc4p derivative specifically designed to target pRB proteolysis; 2) whether the core SCF is in dynamic equilibrium with distinct F-box-containing substrate recognition components, and therefore allows the engineered Cdc4p derivatives to target non-SCF substrates to the core SCF for ubiquitination. As shown in Fig. 7B, the steady state levels of pRB in cells expressing Cdc4p<sup>FWD</sup>-LTP was significantly lower than that in cells expressing Cdc4p<sup>FWD</sup>-LTP(FGSK) incapable of interacting with pRB (Fig. 7B). To further demonstrate that the decrease of pRB levels was a result of stimulated proteolysis by the engineered pRB-specific ubiquitin-protein ligase (Cdc4p<sup>FWD</sup>-LTP), but not due to expression of the pRB binding domain of TAg that affects pRB synthesis, the half life of pRB was determined by promoter shut-off in Y81 cells expressing a second version of engineered Cdc4p with Cdc4p<sup>FWD</sup> fused to the pRB binding domain of the papillomavirus E7 (aa 2-35) (Cdc4p<sup>FWD</sup>-E7N). As a control, the turn over rate of pRB was assessed in Y81 cells constitutively expressing Cdc4p<sup>FWD</sup>-E7N( $\Delta$ DLYC), in which Cdc4p<sup>FWD</sup> was fused to the same domain of E7 internally deleted of four residues ( $\Delta$ DLYC), and therefore lost the pRB binding capability (Munger et al. (1989) EMBO J 8: 40999-105). As shown in Fig. 7C, pRB was rapidly degraded in Cdc4p<sup>FWD</sup>-E7N expressing cells, with a half life of 70 minutes. In Y81 cells expressing Cdc4p<sup>FWD</sup>-E7N( $\Delta$ DLYC) defective for pRB binding, pRB was highly stable within 2 hours following promoter shut-off. These results indicate that by engineering a

pRb binding peptide (LTP or E7N) on Cdc4p, the SCF<sup>Cdc4p</sup> machinery can be directed towards degradation of a non-SCF substrate such as pRb.

**Figure 6** shows a generic scheme for the genetic engineering of the SCF ubiquitin-proteolytic machinery to target degradation of non-SCF substrates (X). Y represents the interaction peptide of protein X that is covalently attached to the F-box-containing SCF subunit to create a "X"-specific ubiquitin-protein ligase.

**Figure 7** demonstrates the trans-targeting of the retinoblastoma tumor suppressor protein. The stable pRB is rapidly degraded by the engineered Cdc4p-derived ubiquitin-protein ligase. **(A)** pRB is a stable protein in *S. cerevisiae*. Exponentially growing Y81 cells carrying PGAL-RB expressing plasmid p2202TRB was induced for 1 hour with 2% galactose. Promoter shut-off was subsequently carried out, and the remaining pRB levels over time were determined from 100 ug of extracts by immunoblotting using the Ab-5 anti-pRB monoclonal antibody (CALBIOCHEM). **(B)** The steady state level of pRB is significantly lower in Y81 cells expressing Cdc4p<sup>F<sup>WD</sup></sup>-LTP than those expressing a mutant Cdc4p<sup>F<sup>WD</sup></sup>-LTP(FGSK) hybrid incapable of binding to pRB. Y81 cells containing pRB and the engineered Cdc4p derivatives, Cdc4p<sup>F<sup>WD</sup></sup>-LTP, or Cdc4p<sup>F<sup>WD</sup></sup>-LTP(FGSK) also under the control of the *GALI,10* promoter, were induced for the indicated hours. Cell extracts were prepared and 100 ug of each extracts were subjected to immunoblotting to detect the steady-state levels of pRB following galactose induction. **(C)** pRB is rapidly degraded in Y81 cells expressing Cdc4p<sup>F<sup>WD</sup></sup>-E7N, but remains highly stable in cell expressing Cdc4p<sup>F<sup>WD</sup></sup>-E7N( $\Delta$ DLYC), which is incapable of binding pRB. The turn over rate of pRB was assessed by promoter shut-off in Y81 cells constitutively expressing Cdc4p<sup>F<sup>WD</sup></sup>-E7N or Cdc4p<sup>F<sup>WD</sup></sup>-E7N( $\Delta$ DLYC), respectively. The decay of pRB over time was determined by immunoblotting using the Ab-5 anti-pRB monoclonal antibody (CALBIOCHEM).

#### Targeted Degradation of the HPV E2 Transcription Factor in Yeast

As a further proof the concept of engineering the SCF<sup>Cdc4p</sup> System, we tested whether Cdc4p could be modified to target degradation of the normally stable E2 transcription factor from the oncogenic human papillomavirus type 16 (HPV16) using the yeast model system. Besides serving as a regulator of viral transcription, E2 also functions as an auxiliary factor that associates with the HPV16 E1 protein directly involved in viral DNA replication (reviewed in Howley (1996) Virology 3rd ed.; New York: Lippincott-Raven Press). The E2 binding domain on HPV16-E1 was mapped to the carboxyl terminal 424-669 amino acids, designated EIC

(Yasugi et al. (1997) Virol 71: 891-9). Furthermore, a Glu39->Ala mutation within the amino terminus of E2 (E2(E39A)) completely abolished E1-E2 association, as determined by the yeast two-hybrid system and *in vitro* binding assays (Sakai et al. (1996); J Virol 70: 1602-11; Yasugi et al. (1997) Virol 71: 891-9). An E2-specific ubiquitin-protein ligase was engineered with the Cdc4p<sup>FWD</sup> segment in-frame fused to E1C and was expressed under the control of the constitutive alcohol dehydrogenase (ADH) promoter in yeast. EE-tagged HPV16-E2 or E2(E39A), which were expressed under the control of the tetracycline-repressible promoter (tetO<sup>R</sup>), were introduced into the wild type INVSC 1 cells together with Cdc4p<sup>FWD</sup>-E1C respectively, and the half lives of E2 or E2(E39A) were determined by promoter shut-off. As shown in Fig. 8, EE-E2 was rapidly degraded, with a t<sub>1/2</sub> of 16 minutes. However, EE-E2(E39A), which was incapable of binding to Cdc4p<sup>FWD</sup>-E1C, remained stable following addition of the deoxycycline (Fig. 8). Therefore, we conclude that the SCF ubiquitination system can be directed towards degradation of specific cellular proteins by covalently attaching their interaction peptides onto Cdc4p. These results provide *in vivo* evidence for a direct role of Cdc4p in substrate recruitment, and further indicate that intact SCFs are transient complexes within the cell. This operational characteristics of the SCF ubiquitination machinery provides enormous flexibility for the core SCF to assemble with a large and diversified family of F-box-containing proteins or their engineered functional derivatives to target degradation of cellular proteins.

**Figure 8** shows that a genetically engineered SCF<sup>Cdc4p</sup> complex efficiently targets degradation of the papillomavirus E2 transcriptional regulator in the yeast model system. INVSc1 cells expressing pADH-Cdc4p<sup>FWD</sup>-E1C and ptetO<sup>R</sup>-EE-E2 or ptetO<sup>R</sup>-EE-E2(E39A) were growing to logarithmic phase respectively. The decay of EE-E2 or EE-E2(E39A) were determined by promoter shut-off with the addition of 10 ug/ml deoxycycline. 10 ml of culture were withdrawn at the indicated time points for preparing protein extracts. EE-E2 or EE-E2(E39A) were detected by immunoprecipitation from 200 ug of extracts followed by immunoblotting using the anti-EE monoclonal antibody (Babco).

#### Targeted Degradation of the Retinoblastoma Tumor Suppressor in Mammalian Cells

A human homolog of the *S. cerevisiae* Cdc4p, h-βTrCP, was recently identified that targets CD4 for degradation in the presence of the HIV1 viral protein Vpu (Margottin et al., 1998). CD4 proteolysis requires the F-box region of h-βTrCP that interacts with human Skp 1, suggesting that an analogous human SCF machinery operates in the degradation of h-βTrCP

substrates (Margottin et al., 1998). Indirect immunofluorescence studies indicated that h- $\beta$ TrCP was localized both in the nucleus and cytoplasm (Fig. 9), suggesting the possibilities that h- $\beta$ TrCP can be engineered to target the degradation of mammalian nuclear and cytoplasmic proteins. To demonstrate the efficacy of the engineered SCF system in mammalian cells to degrade stable cellular proteins, we studied whether expression of an engineered  $\beta$ TrCP-E7N hybrid is capable of mediating proteolysis of the physiologically stable pRB protein in mammalian cells.  $\beta$ TrCP-E7N and  $\beta$ TrCP-E7N( $\Delta$ DLYC) hybrids were constructed in pcDNA3 vector (Invitrogen) and were expressed under the control of the CMV promoter respectively.  $\beta$ TrCP-E7N efficiently interacts with pRB, whereas neither  $\beta$ TrCP-E7N ( $\Delta$ DLYC) nor  $\beta$ TrCP itself was capable of associating with pRB in the *in vitro* binding assays (Fig. 10A). The decay of an exogenous HA-tagged pRB protein was then examined in human osteosarcoma Saos-2 cells lacking a functional pRB. As shown in Fig. 10B, pRB was rapidly degraded in Saos-2 cells expressing  $\beta$ TrCP-E7N, but not  $\beta$ TrCP-E7N ( $\Delta$ DLYC). These results demonstrate that the SCF ubiquitin-proteolytic machinery operates similarly both in yeast and in mammals, and that the F-box-containing ubiquitin-protein ligases can be genetically engineered towards degradation of any cellular proteins of interest.

**Figure 9**  $\beta$ TrCP, a human homolog of Cdc4p, is an unstable protein localized both in the nucleus and in cytoplasm of HeLa cells. **(A)** Human  $\beta$ TrCP is a short-lived protein. The human osteosarcoma Saos-2 cells were transiently transfected with Flag-tagged  $\beta$ TrCP expression plasmid. The decay of F- $\beta$ TrCP over time were determined by pulse-chase analysis by  $^{35}$ S-Express labeling and immunoprecipitation with the anti-Flag M2 monoclonal antibody at the indicated chase times. **(B)** HeLa cells plated on glass coverslips were transfected with Flag-tagged  $\beta$ TrCP (F-TrCP) or control untagged  $\beta$ TrCP. Cellular distribution of the F-TrCP was assayed by indirect immunofluorescence using the anti-Flag M2 monoclonal antibody.

**Figure 10** Degradation of pRB by the engineered  $\beta$ TrCP. **(A)** The engineered F-TrCP-E7N ubiquitin-protein ligase can interact with pRB. *In vitro* translated (IVT),  $^{35}$ S-labeled pRB was mixed with similarly labeled IVTs of  $\beta$ TrCP (lane 1), F- $\beta$ TrCP (lane 2), F-TrCP-E7N (lane 3) and F-TrCP-E7N ( $\Delta$ DLYC) (lane 4) for 1 hour at 4°C. Complexes were then immunoprecipitated using the anti-Flag M2 monoclonal antibody and subjected to SDS PAGE electrophoresis. Lanes 5-9 represent 50% of the input  $^{35}$ S-labeled proteins used in the co-immunoprecipitation analysis. **(B)** The engineered F-TrCP-E7N ubiquitin-protein ligase can degrade transfected pRB. SAOS-2 cells were transiently transfected with the indicated amounts of HA-pRB and F-TrCP-E7N or F-TrCP-E7N( $\Delta$ DLYC). HA-pRB protein was determined from

200 ug of cell extracts by western blotting using the AB-5 anti-pRB monoclonal antibody. (C) F-TrCP-E7N blocks pRB induced growth arrest in SAOS-2 cells. SAOS-2 cells were transfected with 1.5 ug of pBabe-puro and 5.0 ug of each of the indicated plasmids. Transfected cells were subjected to puromycin selection for 7 to 10 days, and the percentage of flat cells were determined as previously described (Tienmann and Hinds (1998) EMBO J 17: 1040-52). The graph shown was the average from two independent experiments.

#### Targeted Degradation of Endogenous p107 in Mammalian Cells

A critical step for the application of the engineered proteolysis system is to target the degradation of endogenous proteins. We therefore tested whether  $\beta$ TrCP-E7N could target the proteolysis of p107, a pocket protein related to pRB and also capable of binding HPV16 E7. The cervical carcinoma C33A cells lacking wt pRB, which can be transfected at high efficiency (60% to 70%), were used in this experiment to study whether endogenous p107 could be degraded by the engineered  $\beta$ TrCP-E7N (Dyson et al. (1992) J Virol 66: 6893-902; Davies et al. (1993) J Virol 67: 2521-8). In this set of experiments, the C33A cells were co-transfected with CMV-CD19, and transfected cells were selected by immunomagnetic selection of those cells expressing CD19. As shown in Fig. 11A, the steady-state levels of p107 were dramatically decreased in  $\beta$ TrCP-E7N expressing cells, but were not affected in C33A cells expressing the control  $\beta$ TrCP-E7N ( $\Delta$ DLYC) protein unable to bind p107. The CD19 negative Saos-2 cells, which did not receive transfected F- $\beta$ TrCP-E7N or  $\beta$ TrCP-E7N ( $\Delta$ DLYC), contain the same levels of p107 (data not shown).

To further improve the efficiency for the delivery of the engineered ubiquitin-protein ligase, we constructed adenovirus expressing F- $\beta$ TrCP-E7N or F- $\beta$ TrCP-E7N ( $\Delta$ DLYC) to infect exponentially growing C33A cells (He et al. (1998) Proc Natl Acad Sci U S A 95:2509-14). Twenty-four hours post-infection, 100% of C33A cells were infected by the viruses, as determined by the expression of green fluorescence protein (GFP) from the adenovirus vector (He et al. (1998) Proc Natl Acad Sci U S A 95:2509-14). As shown in Fig. 11B, expression of F- $\beta$ TrCP-E7N completely eliminated the endogenous p107 in C33A cells, whereas expression of F- $\beta$ TrCP-E7N ( $\Delta$ DLYC) or the control virus has no effect on the cellular p107 protein levels. Collectively, these results demonstrated that by engineering an F-box-containing ubiquitin-protein ligase, the cellular SCF machinery could be redirected to deplete specific cellular proteins through the SCF-mediated ubiquitination machinery.



**Figure 11** shows that expression of the engineered F-TrCP-E7N ubiquitin-protein ligase can target degradation of the endogenous p107 in C33A cells. **(A)** C33A cells were transiently transfected with 3 ug of pCMVCD19 expression plasmid and the pCDNA3 vector alone, or the indicated amounts of pCDNA-F-TrCP-E7N or pCDNA-F-TrCP-E7N ( $\Delta$ DLYC). Following transfection, cells were subjected to immunomagnetic selection by the anti-CD19 monoclonal antibody and the Dynabeads coated with Rat anti-Mouse IgG1. Extracts (200 ug) of CD19 positive cells were examined for the levels of the endogenous p107 using anti-p107 polyclonal antibody (Santa Cruze). The endogenous actin levels are indicated as an internal loading control. **(B)** C33A cells were mock infected, or infected with either control adenovirus **(C)**, or adenoviruses expressing F-TrCP-E7N or F-TrCP-E7N ( $\Delta$ DLYC) for 20 hours to analyze the levels of endogenous p107 as described in **(A)**.

### Experimental Procedures

#### Yeast strains and plasmids.

The *S. cerevisiae* strains used for half life determination and *in vivo* ubiquitination studies were gifts from Drs. D. Finley, S Elledge and S. Reed and were shown as follows: KY130 (*Mat a, ura3-52, leu2- $\Delta$ 1, gcn4- $\Delta$ , ade8::GCN4*), KY203 (*Mat a, ura3-52, leu2- $\Delta$ 1, gcn4- $\Delta$  ade8::GCN4, cdc34-2*), YY371 (*Mat a, ura3, leu2-3, 112, his3-11*), YY374 (*Mat a, ura3, leu2-3, 112, his3-11, prel-1, pre4-1*), Y81 (*Mat a, can1-100, ade2-1, his3-11, -15, leu2-3, -112, trpl-1. ura3-1*), Y553 (*Mat  $\alpha$ , can1-100, ade2-1, his3-11, -15, leu2-3, -112, trpl-1. ura3-1, skpl-11*) or cdc28-4 (*Mat a, ura3, leu2, adel, trpl, arg4, his2, cdc28-4*). SJ1012-4 (*Mat a, leu2-3, -112, ura3-52, lys2, adel, his7, cdc4-5*) is a gift of Drs. B. Byers and B. Jenson. INVSc1 (*Mat  $\alpha$ , his3 $\Delta$ 1, leu2, trpl -289, ura3-52*) was purchased from Invitrogen. Flag-tagged Cdc4p and Skplp, or HA-tagged Cdc53p and Siclp were generated by PCR for cloning into the pYES2 vector (Invitrogen). F-Cdc4p, F-Skplp and HA-Cdc53p were functional since they could complement the temperature sensitive mutants of *cdc4-3*, *skpl-11* and *cdc53-1*. *SIC1-HA* was previously shown to be able to substitute for the chromosomal SIC] (Schwob et al. (1994) Cell 79: 233-44). Flag-tagged Cdc4p carrying the Gly 300 to Asp alteration, or a deletion of the entire F-box, were constructed by PCR-directed mutagenesis and were cloned into the pYES2 vector. Individual Cdc4p segments encompassing the F-box, the WD repeats or both the F-box and the WD repeats were generated by PCR and were cloned into pLDSG5 vector to make fusions to LacZ (Bachmair et al. (1986) Science 234: 179-86). PCR fragments with ubiquitin or Myc-Ub encompassed by the *CUP1* promoter and the *CYCI* terminator sequences (Ellison and

Hochstrasser (1991) J Biol Chem 266: 5966-76) were cloned into the pRS425 vector for coexpression with HA-Grr1p<sup>ΔN</sup> in Y81 cells. All PCR products described above were verified by automated sequencing. Information on details of the plasmid construction are available upon request.

#### Protein stability

Promoter shut-off, pulse-chase analysis, immunoprecipitation and immunoblotting were carried out as described (Bachmair et al. (1986) Science 234: 179-86; Visintin et al. (1997) Science 278: 460-3). For half-life studies by promoter shut-off, exponentially growing KY130 or Y81 cells carrying either F-Cdc4p, HA-Cdc53p, Flag-Skplp, Siclp-HA, F-Cdc4p(G300D), FCdc4p(ΔF) or various Cdc4p segments/β-Gal fusions under the control of the *GAL1-10* promoter, were induced to express the individual proteins for 1 hour by adding 2% galactose to the raffinose containing media. Cells were then transferred to glucose-containing medium containing 1 mg/ml cyclohexamide to repress transcription and translation, respectively (Visintin et al. (1997) Science 278: 460-3). Aliquots from the cell culture were withdrawn at the indicated time intervals, protein extracts were prepared and 100 ug of each extract was used for immunoblotting. To determine Cdc4p stability at different cell cycle stages, KY130 cells carrying GAL-F-Cdc4p were arrested by (x-factor (3 ug./ml) in G1 phase, hydroxyurea (10 mg/ml) in S phase or nocodazole (15 ug/ml) in M phase in raffinose-containing medium. After 3.5 hours, 2% galactose was added to induce synthesis of F-Cdc4p for 45 minutes. Cells were then washed into fresh medium containing 2% glucose and the half life of F-Cdc4p in G1, S or M phase synchronized cells was determined by immunoprecipitation and visualized by immunoblotting using the anti-Flag M2 antibody (Visintin et al. (1997) Science 278: 460-3). For pulse-chase analysis, exponentially growing KY130 cells or Y81 cells expressing HA-Grr1p<sup>ΔN</sup> were pulse-labeled with <sup>35</sup>S-Methionine for 15 minutes followed by chasing in cold fresh medium containing 0.15% L-Cys/0.2% L-Met/50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Portions were withdrawn at the indicated time intervals and analyzed by immunoprecipitation to determine the half lives of the endogenous Cdc4p, Skplp, or HA-Grr1p<sup>ΔN</sup>. To assess Cdc4p degradation in *YY374*, *cdc34-2*, *skpl-11* and *cdc28-4* ts mutants, cells carrying the galactose inducible F-Cdc4p plasmid were grown at 25°C to mid-log phase. Half of each culture was allowed to continue growing at 25°C and the other half was shifted to 37°C for 1 to 3 hours. 2% galactose was then added to induce F-Cdc4p expression in each culture 1 hour prior to promoter shut-off to determine the half-life of F-Cdc4p at the non-restrictive (25°C) or restrictive temperatures (37°C). As a control, the turn

over rate of F-Cdc4p was also determined at 37°C in the respective isogenic wild type cells (data not shown).

### In vivo ubiquitination

Exponentially growing INVSc1 cells expressing an untagged Cdc4p (pYES2) and Myc-Ub (YEP105), under the control of the *GAL1-10* and *CUP1* promoters respectively, were carried out with induction media containing 2% calactose and 100 mM CuSO<sub>4</sub> for 3 hours (Ellison and Hochstrasser (1991) J Biol Chem 266: 21150-7). Yeast cells were lysed by vortexing with glass beads in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris (pH 8.0)), and Cdc4p was immunoprecipitated from 1 mg of each extract adjusted to RIPA buffer condition (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1 % SDS, 50 mM Tris (pH 8.0) ) using 2 ug of affinity purified anti-Cdc4p polyclonal antibody (Harlow (1988) Antibodies, a laboratory manual (Cold Spring Harbor: Cold Spring Harbor Laboratories). The ubiquitinated Cdc4p species was detected by immunoblotting using the anti-Cdc4p or 9E10 antibodies. Y81 cells carrying the constitutively expressed HA-Grrlp<sup>AN</sup> were induced for 3 hours by 100 mM CuSO<sub>4</sub> to express Ub (YEP96) or Myc-Ub (YEP 105) for detection of ubiquitinated Grrlp.

### Yeast strains, cell culture and plasmids

*S. cerevisiae* INVSc1 (Invitrogen) and Y81 cells (gifts of S. Elledge) were used to assess the *in vivo* degradation of the papillomavirus E2 or pRB by the engineered Cdc4p ubiquitin-protein ligases. The human osteosarcoma Saos-2 cell was a gift of Drs. David Thomas and Philip Hinds. The galactose inducible pRB expression plasmid p2202TRB was a gift of Dr. Robert Weinberg (Hatakeyama et al. (1994) Genes Dev. 8: 1759-71). The engineered Cdc4p derivatives designed for targeting pRB degradation were constructed as follows: Cdc4p<sup>F/WD</sup>-LTP and Cdc4p<sup>F/WD</sup>-LTP(FGSK) cDNAs were generated by PCR using the 5' primer 5'-GCGGATCCACCATGGATAAMAAAGAGGGACCTAATAAC-3' that hybridizes to *CDC4* corresponding to residues 270 to 277 with a BamHI site and the ATG translation initiation codon at the 5' end, and the 3' primer encoding sequences of either the Cdc4p C-terminus (residues -779) in frame fused to the pRB binding domain of the SV40 large T antigen (residues 103-115), or the same primer carrying point mutations of the LFCSE motif that abolishes its interaction with pRB (DeCaprio et al. (1989) Cell 58:1085-95; Dyson et al. (1989) Science 243: 934-7; Figge et al. (1993) Protein Sci 2: 155-64; Munger et al. (1989) EMBO J 8: 4099-105); Yang et al. (1995) Nucleic Acids Res 23: 1152-6). The sequences of these two 3' primers carrying the

NotI site and a translation stop codon are 5'-GCGCGGCCGCCTACTCATCATCACTAGATGGCAMCTTCTGAGCAAAACAGCCCTCTGG TATTATAGTTGTCCTCGT-3' and 5'-CGCGGCCGCCTACTCATCATCACTAGATGGCAMTTGAGCCAAAGTTTTCTCTGGTATTATAGTTGTCCTCGT-3'. The resulting PCR fragments encoding Cdc4p<sup>FWD</sup>-LTP or Cdc4p<sup>FWD</sup>-LTP(FGSK) hybrids were digested with BamHI and NotI, and were subsequently cloned into the pYES2 vector (Invitrogen) for expression in Y81 cells under the control of the *GAL1,10* promoter. The Cdc4p<sup>FWD</sup>-E7N or Cdc4p<sup>FWD</sup>-E7N( $\Delta$ DLYC) hybrid constructs were obtained by a two-step PCR approach as described in detail (Dieffenbach, 1995). The first PCR reactions for making individual Cdc4p<sup>FWD</sup>-E7N or E7N( $\Delta$ DLYC) DNA fragments were conducted using pYES-F-CDC4 (this work), pGST-E7(2-35) or pGST-E7(2-35)( $\Delta$ DLYC) plasmids as templates (Gifts of Dr. K. Munger). The sequences for the primer sets used are as follows: 5'-GCWATCCACCATGGATAATITAAAGAGGGACCTAATAAC-3'(5'-extreme) and 5'-GTAGGTGTATCTCCATGTGGTATrATAGTrGTCC-3' for Cdc4p<sup>FWD</sup>, 5'-GGACAACCTATAATACCACATGGAGATACACCTAC-3' and 5'-GCCTCGAGTCACTCCTCCTCTGAGCTGTC-3' (3'-extreme) for E7N or E7N( $\Delta$ DLYC), respectively. The second step PCR reactions were conducted using the same 5'- and 3' extreme primers to ligate Cdc4p<sup>FWD</sup>/E7N or Cdc4p<sup>FWD</sup>/E7N( $\Delta$ DLYC) PCR fragments together. The resulting hybrid Cdc4p<sup>FWD</sup>-E7N or Cdc4p<sup>FWD</sup>-E7N( $\Delta$ DLYC) DNA fragments were digested with BamHI and XhoI which were introduced by the 5'- and 3'- extreme primers, respectively, and were cloned into the p426-ADH plasmid (ATCC) for constitutive expression in Y81 cells under the control of the ADH promoter. Cdc4p<sup>FWD</sup>-EIC were constructed similarly by the two-step PCR approach using primer pairs 5'-GCGGATCCACCATGGATAAMAAAGAGGGACCTAATAAC-3' (5'-extreme)-3' and 5'-CCTATCACATCTATATTTTATTGGTATTATAGTTGTC-3' for Cdc4p<sup>FWD</sup>, and 5'-GACAACCTATAATACCAATAAAATATAGATGTGATAGG-3' and 5'-GCCTCGAGTCATAATGTGTGTAGTATTTTGTCTG-3' for E1C. The resulting Cdc4p<sup>FWD</sup>-EIC fragment was cloned into the BamHI/XhoI sites of p426-ADH vector. EE-tagged HPV16-E2 and HPV16-E2(E39A) were generated by PCR using primers 5'-GCGGATCCACCATGGAGGAAGAAGAGTATATGCCCA TGGAGGAGACTCTT TGCCAACGTTTTAAATGTG-3' and 5'-GCGCGGCCGCTCATATAGACATAAATCCA GTAGAC -3', and the resulting PCR fragments were cloned into the single copy plasmid pCM185 for expression under the control of the tetracycline-repressible (tetO<sup>R</sup>) promoter in *S. cerevisiae*.

$\beta$ TrCP-E7N and  $\beta$ TrCP-E7N( $\Delta$ DLYC) were also constructed by the two step PCR approach with primer pairs 5'-GCGGATCCGCCACCATGGACTACAAGGACGACGATGACAAAGATGACCCGGCCGAGGCGGTGCTG-3' and 5'-GTAGGTGTATCTCCATGTCTGGAGATGTAGGTGTATG-3' for PTRCP, 5'-CATACACCTACATCTCCAGACATGGAGATACACCTAC-3' and 5'-GCGCGGCCGCTCACTCCTCCTCTGAGCTGTC-3' primer sets for E7N or E7N( $\Delta$ DLYC). The final PCR fragments were cloned into the BamHI/NotI sites of pcDNA3 (Invitrogen). The cloned PCR fragments described above were sequenced by the BCMP core facility at Harvard Medical School. Expression of the engineered Cdc4p hybrids in Y81 cells were confirmed by immunoblotting using the anti-Cdc4p polyclonal antibody.

#### Immunofluorescence staining

The cellular distribution of  $\beta$ TRCP was analyzed by indirect immunofluorescence. Hela cells grown on glass coverslips were transfected with F-TrCP,  $\beta$ TRCP or pCDNA3 vector using the FuGENE transfection reagent. 36 hours post transfection, cells were washed with PBS and fixed in 3% paraformaldehyde for 10 minutes at room temperature. Cells were then washed in blocking buffer (0.2% Fish Skin Gelatin, 0.02% Triton-100 in PBS) and hybridized with the anti-Flag monoclonal antibody (3 ug/ul) (Kodak) at 1:500 dilution in blocking buffer for 30 minutes. After washing 3 times with blocking buffer, cells were hybridized with 1:500 dilution of Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc.) for 30 minutes, washed twice in blocking buffer, once in PBS, and mounted on microscope slides for visualization.

#### Interaction of pRB with the engineered ubiquitin-protein ligases

Interaction of pRB and the pRB binding peptides for engineering Cdc4p-derived ubiquitin-protein ligases were demonstrated previously by *in vitro* binding and by the two hybrid interaction assay in yeast cells (Yang et al. (1995) Nucleic Acids Res 23: 1152-6 (K. Munger, personal communications). To verify the interaction between pRB and the engineered F-TrCP-E7(N), pRB and the individual F-box/WD40 repeats-containing  $\beta$ TrCP derivatives were synthesized *in vitro* in the TNT rabbit reticulocyte lysates in the presence of  $^{35}$ S-methionine. 10 ul of each radiolabeled proteins,  $\beta$ TrCP, Flag-tagged  $\beta$ TrCP (F-TrCP), F-TrCP-E7(N) and F-TrCP-E7(N)( $\Delta$ DLYC) (lanes 1-4) were mixed with 10 ul of  $^{35}$ S-labeled pRB individually for co-immunoprecipitation with 1 ug of the anti-Flag (M2) monoclonal antibody (Kodak) in NP40 lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM Tris-HCl, 5 mM EDTA, 1 mM

dithiothreitol, 0.01% phenylmethylsulfonyl fluoride, 1 ug each of aprotinin and leupeptin) as described in detail (Harlow (1988) Antibodies, a laboratory manual (Cold Spring Harbor: Cold Spring Harbor Laboratories), and were electrophorized on a 7.5% SDS PAGE gel.

#### Engineered SCF system in yeast and mammalian cells

To evaluate pRB stability in *S. cerevisiae*, Y81 cells containing the pRB expressing plasmid p2202TRB under the control of the *GAL1,10* promoter were grown to log phase in raffinose-containing medium, and were induced for 1 hour by addition of 2% galactose. Cells were washed into glucose-containing medium in the presence of 1 mg/ml cyclohexamide to repress pRB transcription and translation respectively. Aliquots of cells were collected at the indicated time points following promoter shut-off, extracts were prepared by vortexing in glass beads containing NP-40 lysis buffer, and 100 ug of each extracts were analyzed by immunoblotting using the Ab-5 anti-pRB monoclonal antibody (CALBIOCHEM). pRB half life was determined similarly by promoter shut-off in Y81 cells constitutively expressing the engineered Cdc4p derivatives, Cdc4p<sup>FWD</sup>-E7N or Cdc4p<sup>FWD</sup>E7N( $\Delta$ DLYC), under the control of the alcohol dehydrogenase (ADH) promoter. The steady-state levels of pRB was also determined from 100 ug of Y81 extracts induced for the indicated hours to express Cdc4p<sup>FWD</sup>-LTP or the mutant Cdc4p<sup>FWD</sup>-LTP(FGSK) hybrid by immunoblotting using the Ab-5 anti-pRB monoclonal antibody (CALBIOCHEM). The half lives of EE-E2 or EE-E2(E39A) were similarly determined in Cdc4p<sup>FWD</sup>-E1C expressing INVSc1 cells by promoter shut-off in the presence of 10 ug/ml deoxycycline, a metabolically stable tetracycline derivative. The decay of EE-E2 or EE-E2(E39A) over time were determined by immunoprecipitation followed by immunoblotting using the anti-EE monoclonal antibody (Babco).

To demonstrate the efficacy of the engineered ubiquitin system in mammalian cells, the human osteosarcoma Saos-2 cells lacking endogenous pRb were co-transfected with 5ug each of HA-tagged pRB and F-TrCP-E7(N) (lanes 1-3), or with 5ug each of HA-pRB and F-TrCP-E7(N)( $\Delta$ DLYC) (lanes 4-6) using the FuGENE transfection reagent, respectively. 36 hours after transfection, cells were starved for 1 hour in DMEM media without L-Met and L-Cys, pulse labeled for 1 hour with the <sup>35</sup>S-Express labeling mix, and then chased with fresh DMEM media containing 15% fetal calf serum at times 0, 3 hours and 6 hours. Cell lysates were prepared in NP-40 lysis buffer and 200 ug of each extracts were subjected to immunoprecipitation by the 12CA5 monoclonal antibody.

**6. Polypeptide and Nucleic Acid Compositions****F-Box Polypeptides and F-Box Encoding Nucleic Acids**

The F-box protein Cdc4p is encoded by a nucleic acid sequence corresponding to nucleotides 7558 to 9897 of GenBank Accession No.D31600 (SEQ ID No. 1), and which encodes the F-box protein corresponding to GenBank Accession No. BAA06495 (SEQ ID No. 2).

The human F-box protein h $\beta$ TrCp is encoded by a nucleic acid sequence corresponding to GenBank Accession No. NM\_003939 (SEQ ID No. 3), and which encodes the F-box protein corresponding to GenBank Accession No. 4502477 (SEQ ID No.4).

The yeast F-box protein Grr1p is encoded by a nucleic acid sequence corresponding to GenBank Accession No. M59247 (SEQ ID No. 5), and which encodes the F-box protein corresponding to GenBank Accession No. AAA34652 (SEQ ID No.6).

The yeast F-box protein Met30p is encoded by a nucleic acid sequence corresponding to nucleotides 3742 to 5664 of GenBank Accession Nos. Z46861 and Z47047 (SEQ ID No. 7), and which encodes the F-box protein corresponding to GenBank Accession No. CAA86905 (SEQ ID No.8).

The fission yeast F-box protein Pop2 encodes a novel F-box/WD-repeat protein involved in the proteolysis of the Cdc2p inhibitor Rum1p and the replication initiator Cdc18p and is encoded by a nucleic acid sequence corresponding to GenBank Accession No. AF038867 (SEQ ID No. 9), and which encodes the F-box protein corresponding to GenBank Accession No. AAB95480 (SEQ ID No.10).

The murine F-box protein FWD1p involved in ubiquitin-dependent degradation of IkappaBalpha and is encoded by a nucleic acid sequence corresponding to GenBank Accession No. AF081887 (SEQ ID No. 11), and which encodes the F-box protein corresponding to GenBank Accession No. AAD17755 (SEQ ID No.12).

Other exemplary polypeptides and nucleic acids for use in the invention include:

Exemplary SCF subunit-encoding genes and their encoded polypeptides include Skp1p (GenBank Accession No.U61764) which encodes Skp1p (GenBank Accession No. AAC49492); and the murine SCF complex protein cul-1 (encoded by the nucleic acid of GenBank Accession No.AF083216 which encodes the cul-1 polypeptide of GenBank Accession No. AAD16038).

### **Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.



**We claim:**

1. A method for targeting degradation of a polypeptide in vivo comprising:  
providing a ubiquitin protein ligase polypeptide that encodes a ubiquitin conjugation activity, which is functionally linked to a target polypeptide interaction domain that provides a target polypeptide recruitment activity;  
expressing said ubiquitin protein ligase polypeptide-target polypeptide interaction domain hybrid in a cell;  
recruiting the target polypeptide to said ubiquitin protein ligase polypeptide; and  
ubiquitinating the target polypeptide,  
thereby forming a ubiquitin-target polypeptide conjugate which is targeted for degradation.
2. The method of claim 1, wherein the ubiquitin-target polypeptide conjugate further undergoes ubiquitin-dependent proteolysis.
3. The method of claim 2, wherein said ubiquitin-dependent proteolysis is by the proteasome.
4. The method of claim 1 wherein the ubiquitin protein ligase polypeptide is an E3 ubiquitin protein ligase.
5. The method of claim 4, wherein the E3 ubiquitin protein ligase is selected from the group consisting of: an SCF polypeptide, a HECT polypeptide, and a UBR1 polypeptide.
6. The method of claim 5, wherein the SCF polypeptide is an F-box polypeptide.
7. The method of claim 6, wherein the F-box polypeptide further comprises a WD domain.
8. The method of claim 6, wherein the F-box polypeptide is selected from the group consisting of Cdc4p, Pop1p, Pop 2p, Grr1p, Met30p, HOSp, beta TrCPp, and FWD1p.
9. The method of claim 6, wherein the F-box polypeptide is a polypeptide selected from the group consisting of SEQ ID Nos. 2, 4, 6, 8, 10, and 12.

10. The method of claim 6, wherein the F-box polypeptide is encoded by a nucleic acid selected from the group consisting of SEQ ID Nos. 1, 3, 5, 7, 9, and 11.
11. The method of claim 5, wherein the SCF polypeptide is selected from the group consisting of: a Cdc4 polypeptide, a Skp1 polypeptide or a cullin polypeptide.
12. The method of claim 5, wherein the HECT polypeptide is selected from the group consisting of E6-AP, Nedd-4, RSP5, Smurf1, TOM1 and EDD.
13. The method of claim 5, wherein the E3 ubiquitin protein ligase is a yeast or a mammalian UBR1 polypeptide.
14. The method of claim 1, wherein the target polypeptide interaction domain is selected from the group consisting of a papillomavirus E7 polypeptide, and an SV40 LTP polypeptide.
15. The method of claim 1, wherein the target polypeptide is selected from the group consisting of a retinoblastoma polypeptide, a p107 polypeptide, IκB, Sic1p, Cln2p, E2 or beta-catenin.
16. A method for decreasing the level of a target polypeptide comprising:  
providing an SCF recruitment domain which is operably linked to a target polypeptide interaction domain to form an SCF recruitment domain-target polypeptide interaction domain fusion protein; and  
expressing the SCF recruitment domain-target polypeptide interaction domain fusion protein such that the level of said target polypeptide is decreased.
17. The method of claim 16, wherein the SCF recruitment domain is an F-box polypeptide.
18. The method of claim 17, wherein the F-box polypeptide further comprises a WD domain.
19. The method of claim 17, wherein the F-box polypeptide is selected from the group consisting of Cdc4p, Grr1p, Pop1p, Pop 2p Met30p, HOSp, betaTrCp, and FWD1.

20. The method of claim 17, wherein the F-box polypeptide is at least 70% to a polypeptide of at least 20 contiguous amino acids of a polypeptide selected from the group consisting of SEQ ID Nos. 2, 4, 6, 8, 10, and 12.
21. The method of claim 17, wherein the F-box polypeptide is encoded by a nucleic acid which hybridizes to a nucleic acid selected from the group consisting of 1, 3, 5, 7, 9, and 11.
22. A method for creating a destabilized polypeptide subject to SCF-mediated proteolysis comprising:  
providing an SCF recruitment domain; and  
operably linking the SCF recruitment domain to the polypeptide, thereby creating the destabilized polypeptide subject to SCF-mediated proteolysis.
23. A method for expressing a destabilized target polypeptide subject to SCF-mediated proteolysis comprising:  
providing an SCF recruitment domain which is operably linked to a target polypeptide and expressing the SCF-target polypeptide fusion, thereby expressing a destabilized target protein.
24. A nucleic acid for expressing an SCF recruitment domain- target polypeptide interaction domain comprising:  
a nucleic acid encoding an SCF recruitment domain; and  
a nucleic acid encoding a heterologous polypeptide domain;  
wherein the nucleic acid encoding the SCF recruitment domain and the nucleic acid encoding the heterologous polypeptide domain are operably linked so as to encode an SCF recruitment domain-heterologous polypeptide domain fusion protein.
25. The nucleic acid of claim 24, wherein the heterologous polypeptide domain is a target polypeptide.

26. The nucleic acid of claim 24, wherein the heterologous polypeptide domain is a target polypeptide interaction domain.
27. The nucleic acid of claim 24, wherein the nucleic acid encoding the SCF recruitment domain is at least 90% identical to a nucleic acid selected from the group consisting of SEQ ID Nos. 1, 3, 5, 7, 9, and 11.
28. The nucleic acid of claim 24, wherein the nucleic acid encoding the SCF recruitment domain hybridizes under stringent conditions to nucleic acid selected from the group consisting of SEQ ID Nos. 1, 3, 5, 7, 9, and 11.
29. A vector comprising a nucleic acid encoding an SCF recruitment domain and a cloning site for inserting an heterologous polypeptide encoding sequence.
30. A vector comprising a nucleic acid of claim 24.
31. A cell comprising a vector of claim 29 or claim 30.
32. A method of treating a cell to stabilize a target polypeptide of ubiquitin protein ligase comprising contacting the cell with a preparation comprising an effective amount of an organic compound which can competitively inhibit interaction of the target polypeptide with the ubiquitin protein ligase.
33. The method of claim 32, wherein the organic compound is a peptide or peptidomimetic.
34. The method of claim 33, wherein the peptide or peptidomimetic is a competitive inhibitor of a WD domain.
35. The method of claim 33, wherein the peptide or peptidomimetic comprises a general chemical formula of  $G-H-X^{(3-6)}-h-X-X-h-X-r-X-t^{(2-3)}-p-X-h-h-X-X-X-X-D-X-X-X-X-h-W-D$ .

1/12



Fig. 1A



Fig. 1B

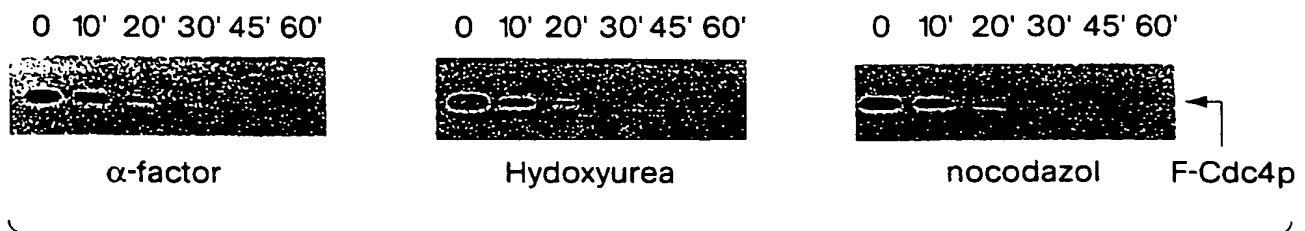


Fig. 1C

SUBSTITUTE SHEET (RULE 26)

2/12

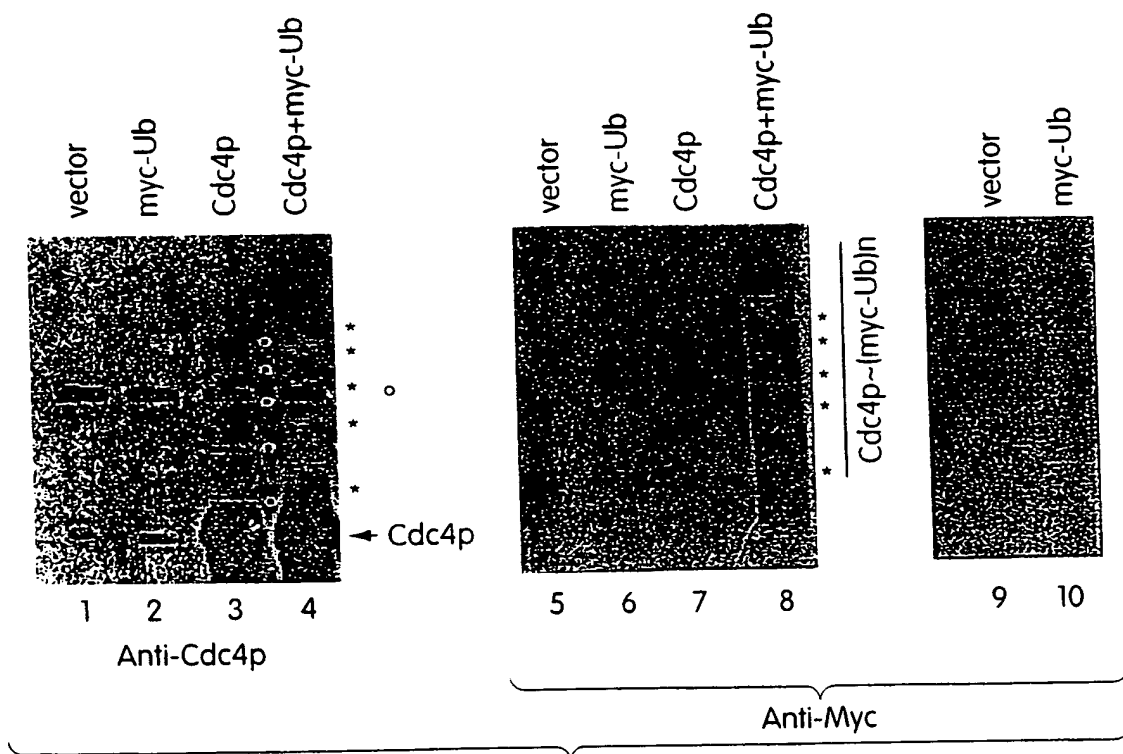


Fig. 2A

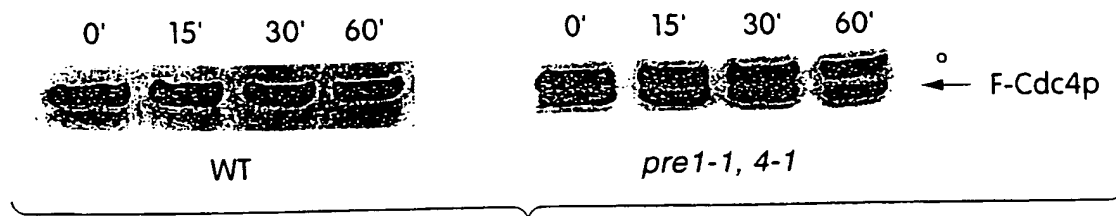


Fig. 2B

3/12

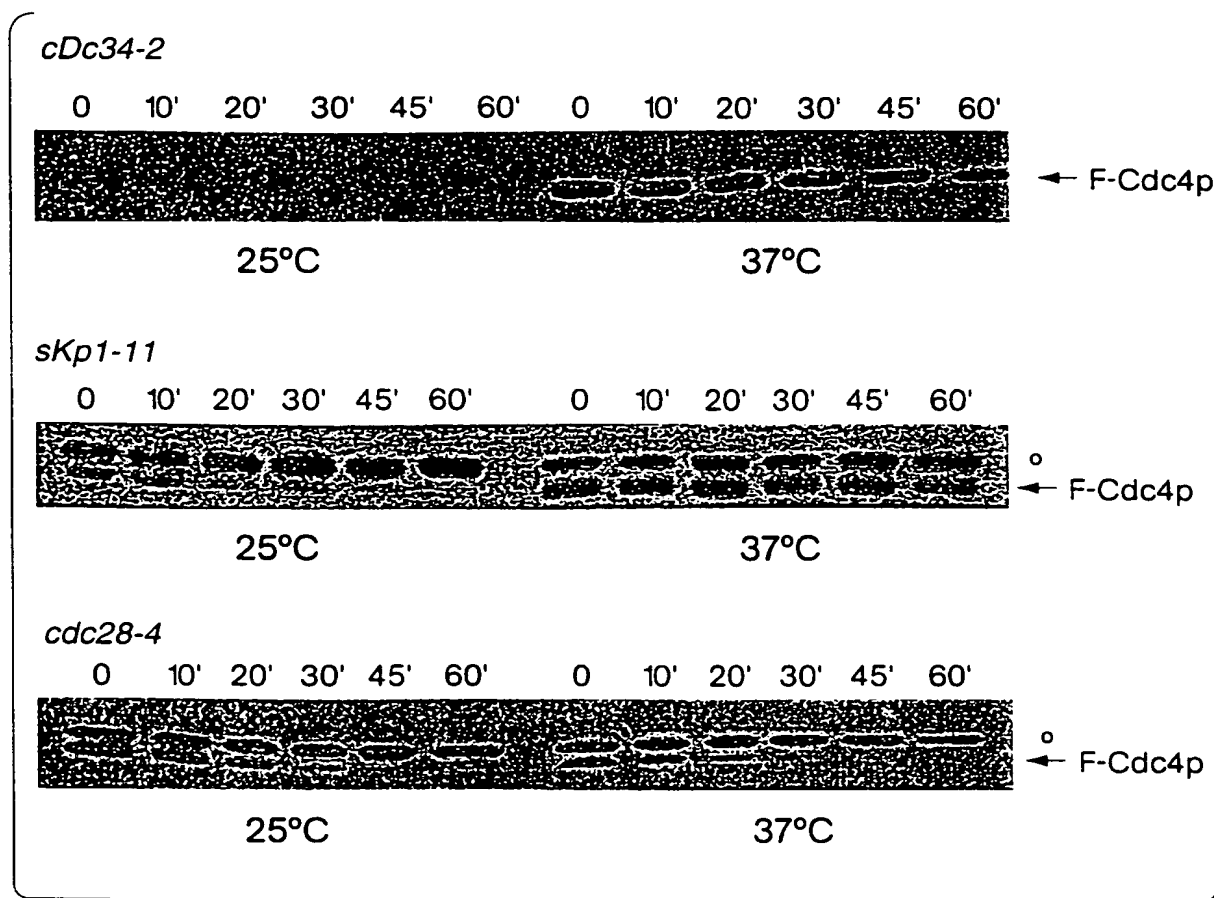


Fig. 3

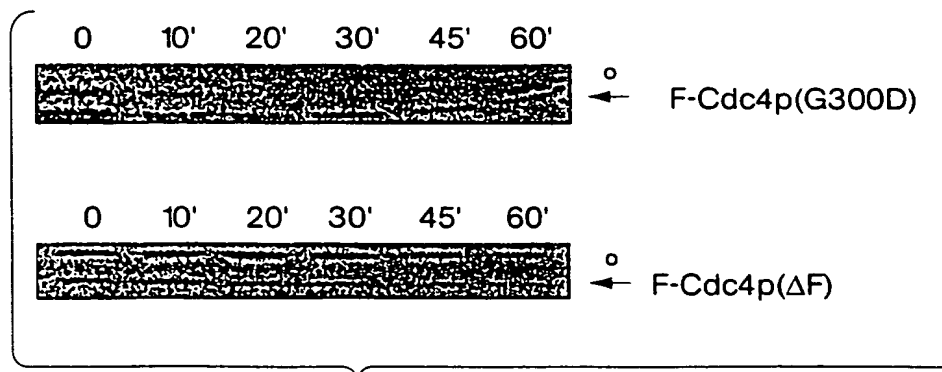


Fig. 4A

SUBSTITUTE SHEET (RULE 26)

4/12

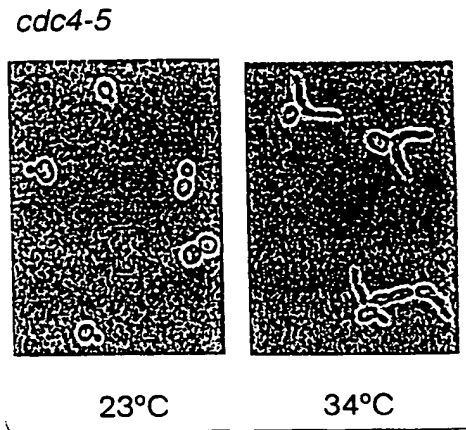


Fig. 4B

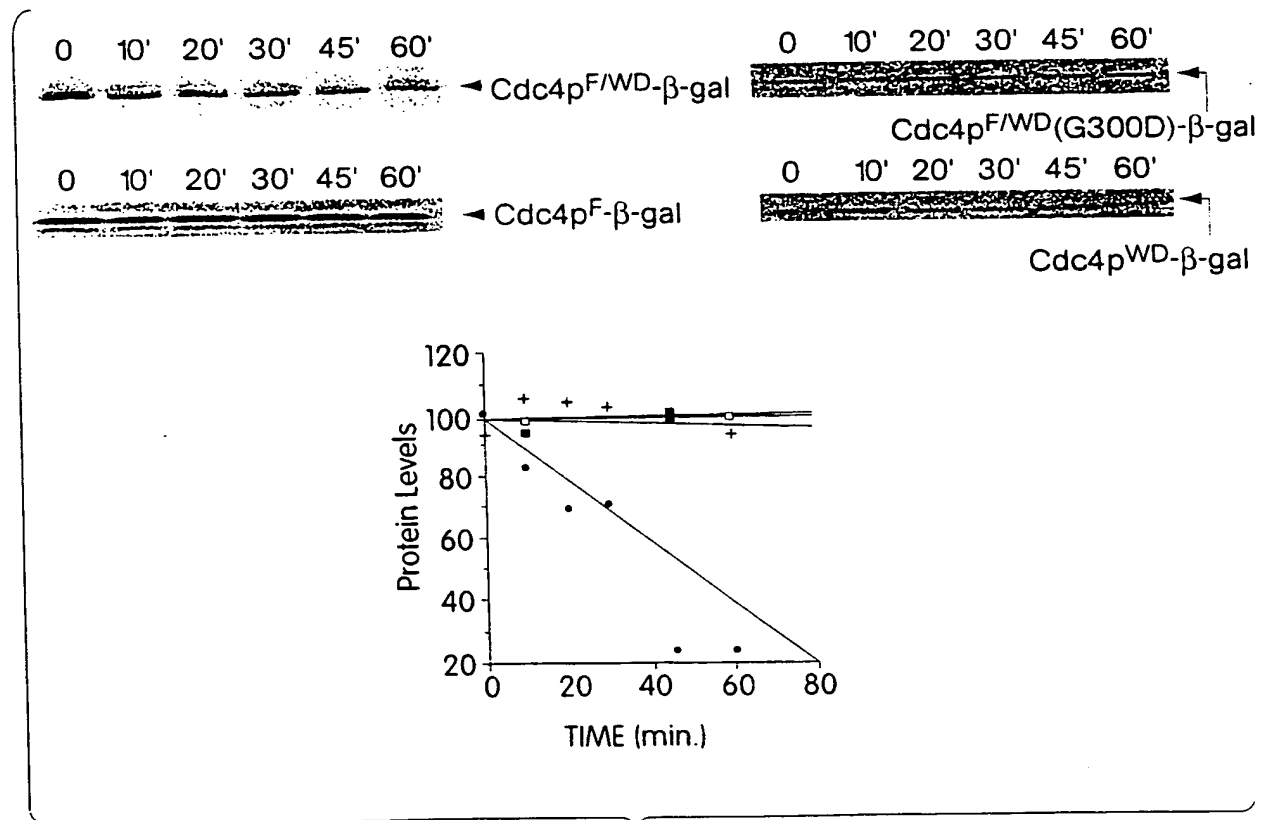


Fig. 4C

SUBSTITUTE SHEET (RULE 26)



5/12

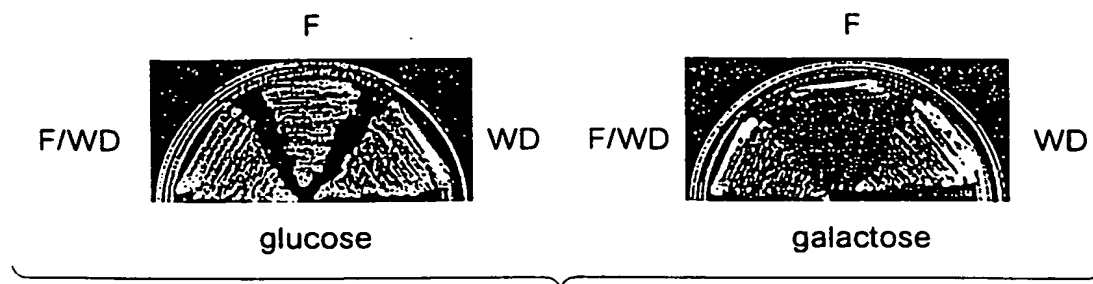


Fig. 4D

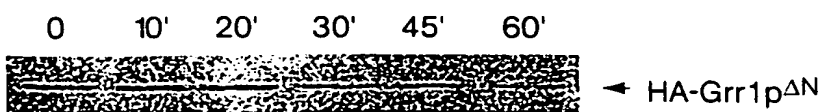


Fig. 5A



Fig. 5B

SUBSTITUTE SHEET (RULE 26)

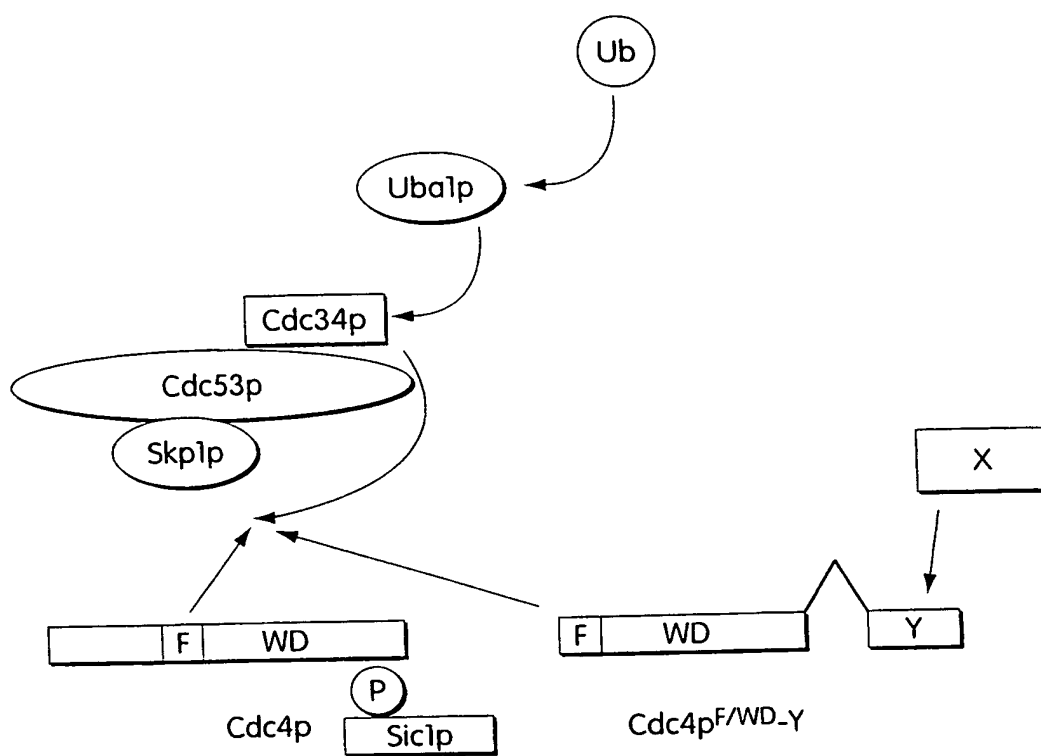


Fig. 6

7/12

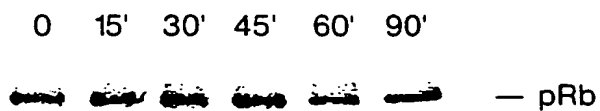


Fig. 7A

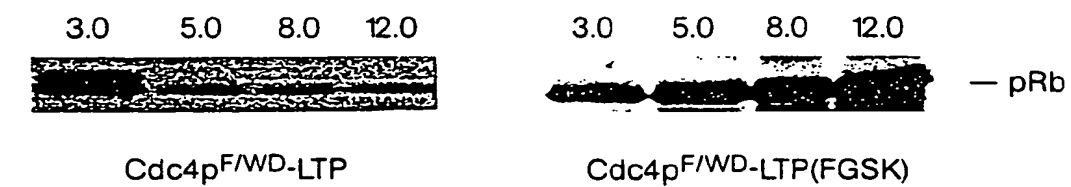


Fig. 7B

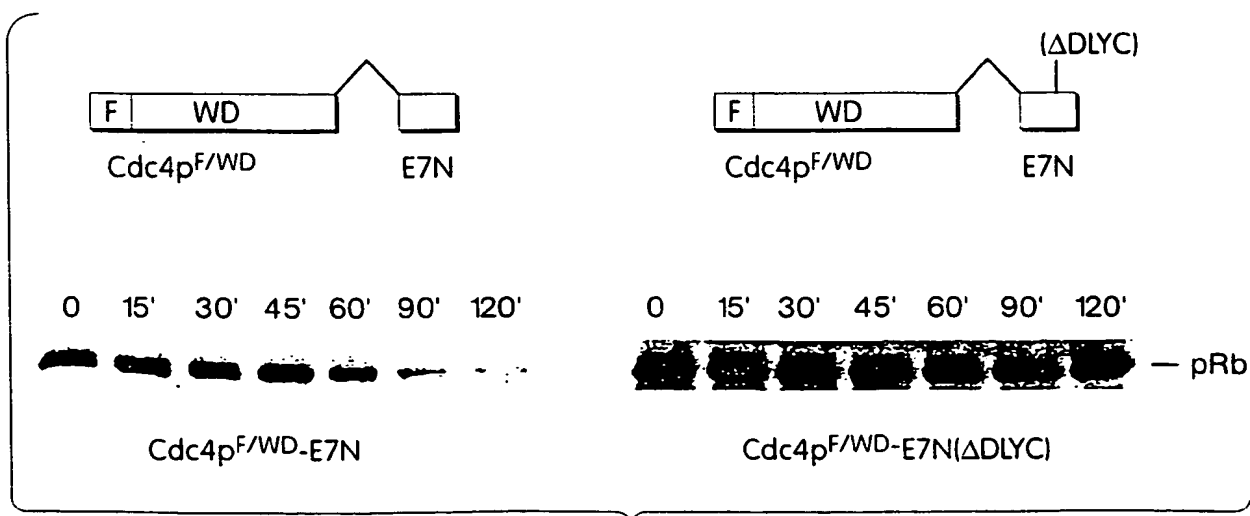


Fig. 7C

SUBSTITUTE SHEET (RULE 26)

8/12

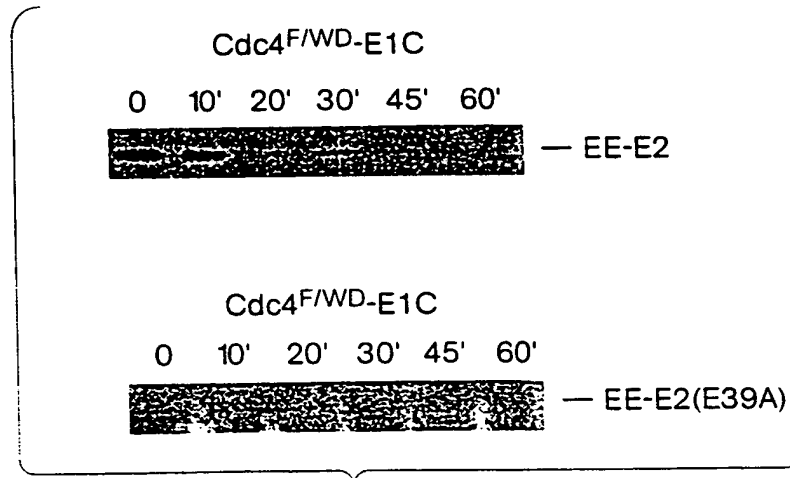


Fig. 8

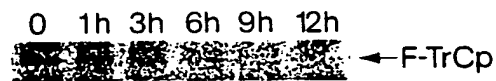


Fig. 9A



Flag-βTrCP



βTrCP

Fig. 9B

SUBSTITUTE SHEET (RULE 26)

9/12

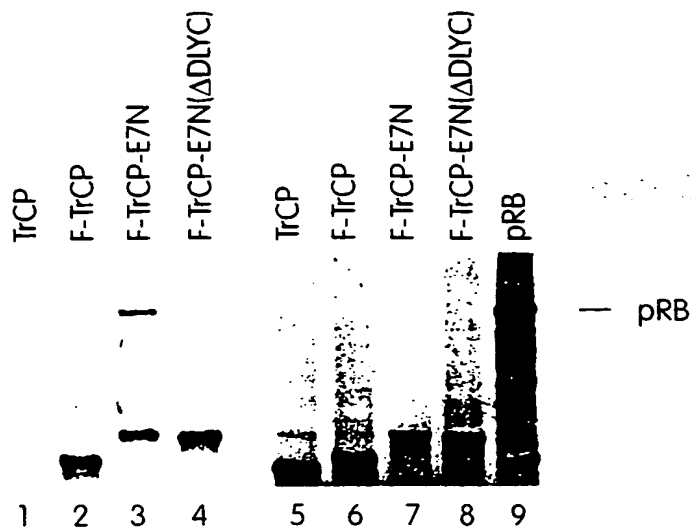


Fig. 10A

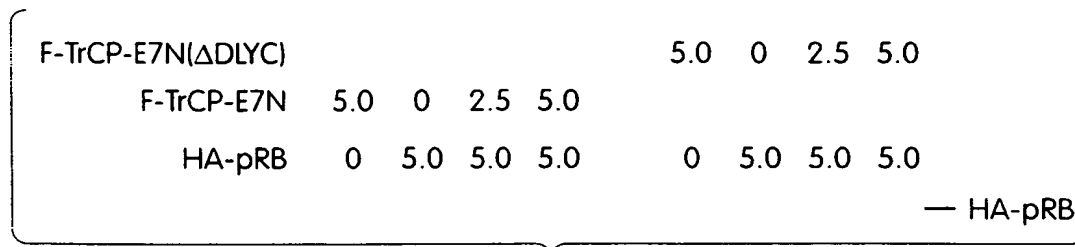


Fig. 10B

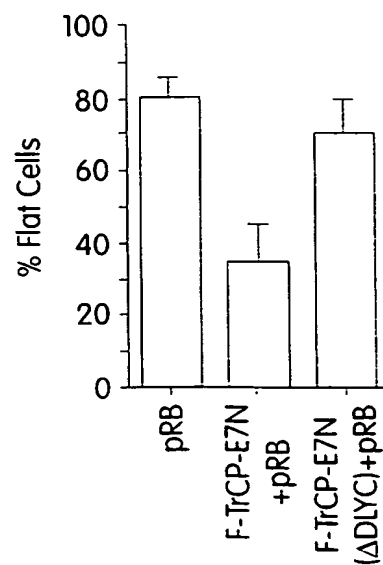


Fig. 10C

SUBSTITUTE SHEET (RULE 26)

10/12

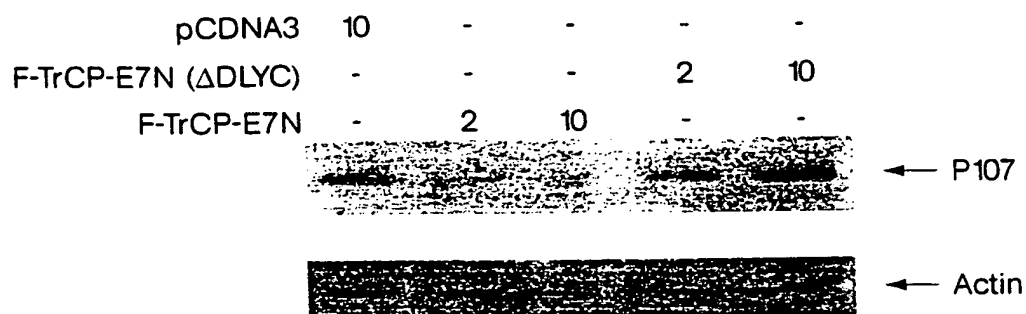


Fig. 11A

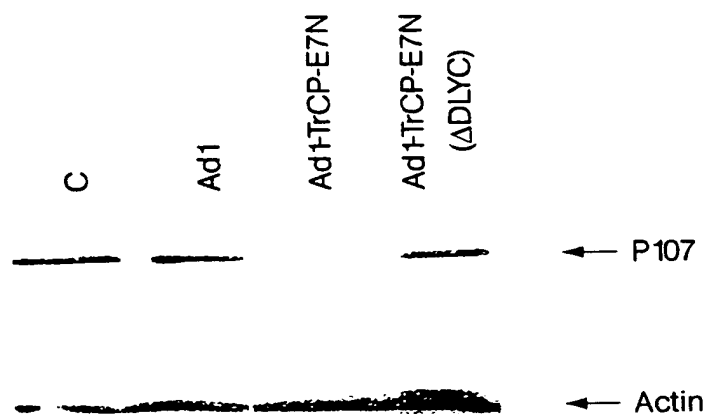


Fig. 11B

SUBSTITUTE SHEET (RULE 26)

11/12

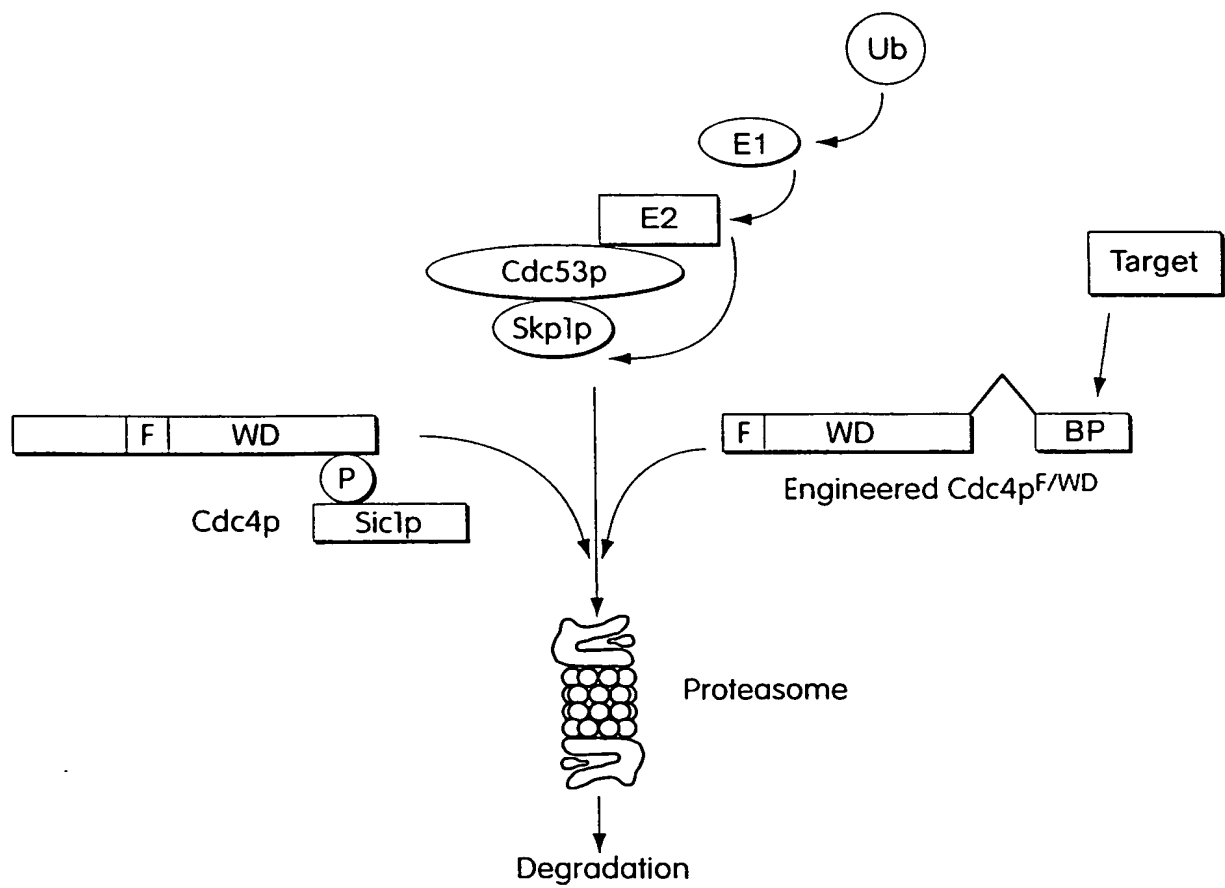


Fig. 12A

12/12

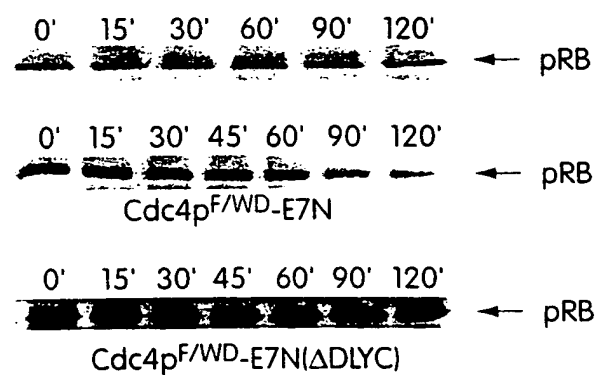


Fig. 12B

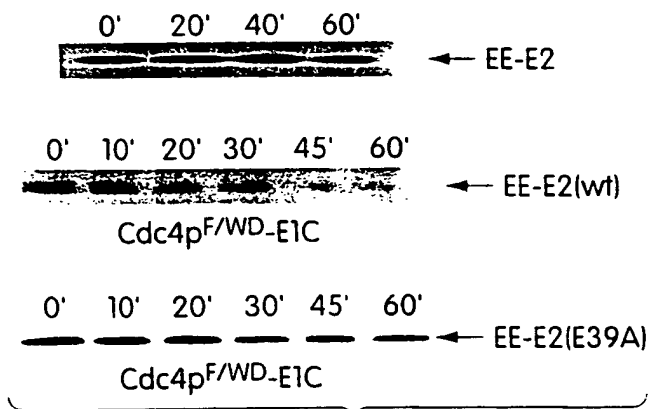


Fig. 12C



SEQUENCE LISTINGSEQ ID No. 1

```

1  ggatccgtgt  cgtaactgcg  ttccgtacac  cttaaaatga  actttccagc  aggtgcacca
61  tcttccaatc  cattgaaaaa  tcttactgat  tcttcgacct  cctcctggct  taagtttgtc
121  tccaaatgaa  tatatagcag  ccaaatcaat  atgtttgatc  tatcctttgg  ctcgattttc
181  gaatagtcca  gtatatcaaa  taatgttggt  gcataagctg  cttttgattc  ttgtttttca
241  ttttccgctt  ccgtttcttt  tacttcattc  tgttcatcta  ccatatcttg  ctctgagctt
301  ttcgattcga  cttccacata  cttggaaaaga  aacctcttgt  caattaggcc  ggaattatca
361  catatgctaa  acaacatatt  aataatgtta  aatttgtaa  cgggtgtccac  ttcaccatca
421  ttttggtcga  cctttttgta  agtctccatc  aatgagagtt  gctcattgcc  ccacggcaga
481  tttttcagaa  tagatttcag  tctaggtgta  tcctgcaaaa  gtttcggatc  attggcatgt
541  aattgtaaaa  ctggcacgga  gtgaaaaagt  ctcaattgcy  atgtcatttc  taaataaaaa
601  tttatagtgg  tgttcaacct  accaatgttg  accaataacg  ctaaaacaca  agtagaaaat
661  gctacttggt  ggtctaataa  tagtttatct  ctgagaattt  tggaacattt  gatagaagtg
721  gctattgtca  acacatacaa  ttgtgcgaac  gtcaatttat  cgtaaataac  aaatgtcctt
781  gcatcgctac  ctttgtcggt  cacgttgatt  gataatttat  cgccatcgat  attaatgggt
841  acaatagagt  tcttgatatg  ggggttttaga  acattagtga  agatttgcc  ctgtccatc
901  aacagttctt  gcatgaaact  gaactgaatg  tcctttctat  tgaatggctc  gccgtcaggt
961  ttcttcaaat  gacgattgta  ttactggca  ttgctttctt  tgggtttcct  tctaacagtg
1021  ttagttgtca  ctggccccgt  tgccgtactt  gtcctgttg  cttcaggcac  tgtagaaggt
1081  tcgaagtga  gcaagatggt  ggtattttct  tcttcacttt  gcgtggagtc  atcatcattt
1141  tctcgtcga  tttcaatctc  gtgtataact  ggaatgcttt  ttgtagtcac  agtctcggtc
1201  tctgattgaa  tggagcttgg  ggatgcttct  tcttgatc  ccgattttac  agattgcatt
1261  ggagaatctg  cttttgtttc  gtcagaattg  tcttctcgta  gttggaaagt  atcatgaatt
1321  ggatcgata  ctctcttccc  catctttaat  tagagtgtcg  cttttattta  gctttcgatt
1381  ttacttagtt  aatcatgaac  tgtttccaat  accatatcag  cattcattgg  gcgttcttta
1441  cttacttgta  ccgttaccgc  attcgagggt  aacctgtttt  tcgttgta  cgatatgcta
1501  cagaattgtc  ttaatatggg  ctaagaaaaa  aaaaagtctg  attatttctg  atactgcaaa
1561  atataactg  gcttggttaa  gaaaagtgtt  gctttagttc  ctttacatca  agagtcgtta
1621  aattgttttg  tgtcataaca  gtatgggtct  tttagacat  caatcgactc  tcaggttttc
1681  cttctgttca  tcttctgtt  ttcggttaa  ttcagccagc  atatttcaat  tctctctttt
1741  ttgaaataac  attatgtaat  ctttcaatcc  tttctatcca  acgtctgcta  aataatttat
1801  tcaaatgaca  tgtagaccag  aaaatcgaac  gttggataat  ttttcactct  gaatcatggt
1861  ggtctcaact  agagcaattt  tttcacgtcc  tctgacacaa  aactcgataa  actgtcgcac
1921  cagtttaaaa  aaatatgtac  gtgtataata  actgaattta  aaaggatagt  aaataatttt
1981  tgcattttat  ggaatgtcaa  aatactaata  agtcgcaaat  atagctatca  aataccataa
2041  ttagctact  tatagaaaga  tgcccaaact  ccgacaaaaa  aggaccattg  cgtcttcctc
2101  gtcagttttt  tatggaagtt  cacctttcca  aaatgatggc  tacatcaaag  taatggaact
2161  cgtatcacac  attgtcattg  aaataaatca  ttcacctacc  gcaacaacgg  atgaaacgag
2221  aaagcagaat  aatccggagc  tgaaagtga  agaaccagt  tgaacctca  agaagtggga
2281  aaataacact  aactttatat  tggagatca  tacgaagaat  aaaacaaaac  tttctagcac
2341  agataggata  cgtaagtggg  ttagaagaca  tatattaaag  gaagagatcg  aaatcctttc
2401  ccatggaaaa  caattgagta  gtattgatga  ggattattgc  ctttcaaatg  ttcttgtagg
2461  atgttcaaga  gatctaaata  aactcagatc  atttcaaaat  ttttaggaat  ataaaatttc
2521  tcagtaagaa  aaagaagatg  aatgcgttta  tagtatttta  agttgttcaa  tagaatatct
2581  tcaatatggt  catatgtgga  ctaaaagaag  attttggtat  atgtctataa  atttattggt
2641  ggtaaacgcc  attaagcagc  tttccaagaa  tatgttatca  ttgaggagaa  gtacgaaaac
2701  tgattatgca  cgattaggaa  aaattctgca  aggagtatta  aaaaatggcc  ttcgagaaga
2761  aatgaaatct  ctttcgatga  gcaattggtc  tatggataat  ttgatagaca  tatgaaaaag
2821  tcaaaagtgt  ttttgcccat  atgcagtaaa  aacttaccct  tcaaatgca  aaaccattag
2881  ttttattata  tttttttttt  tttctttcat  tgaagtatac  acgaaatccc  ataccgaaat
2941  aaacagtcgt  tgtatcatca  atttcggctg  tctggatggc  gactacgaga  ggaagcttgc
3001  ttgccatgat  aggaccaatc  tcaattttta  atttttcatt  aactagctaa  ttttctataa
3061  tgctctaagt  ttcttttttc  gttttgtttc  tttatagtga  tgatgctgtt  attgtgacgc
3121  tatcaaatca  tatactgtca  tcaatcctca  tctcatcggc  tccggaacgc  gttgaaatag
3181  ttgttaaaca  agctctttat  agctagctat  agtagaaaag  gaaggaaaga  agtctaactg
3241  ctgtattatc  gactgacaag  ggtgtgcagt  tgccttcttt  tattttttgt  actccctgga

```

SUBSTITUTE SHEET (RULE 26)

```

3301 atcgagcgga tgtatttttt aacaaagtat tcagaattgt ttttgtataa tcaaaatttg
3361 tcgacgctaa gaaaatgacg gacgtcgtaa ttcgctact aggttttgtg tgctcatcga
3421 tatttgacac ctatttttga cacttttaag gttatatact ttgtttcggg tggtgggtac
3481 actcagttga ctgattatga aagatatggc gtagccgttg ggtaggggtt attacacaga
3541 aatgaattat accagtgaat ttattctctt tatgtttgtt cctacatgtt ttccaatcag
3601 aaggaattgt tatataagga cacaagaaat gtgttggtac gctctgcgcc tcaatgacgg
3661 tttatctatc taaaactatt tctttctcgt aataaaaaata caataaataa ttaataataa
3721 tatagttgat cgatataaca aaaaaataac agcgaggatg gttagttcaa gtgtttccat
3781 tttggggact agcgccaagg catccacttc tctaagtaga aaggatgaaa ttaactaac
3841 ccctgaaaca agggaagcta gcttgacat tccatacaaa cccattattg catactggac
3901 ggtgatgggt ctctgtctga tgattgcctt tgggtggattc atttttgggt gggatacagg
3961 aaccatttca ggggtttatta accaaacaga tttcaagaga aggtttgggt agttacaaag
4021 ggacggcagt tttcaactat cagatgtcag gacagggcta attgtcggta tcttcaacat
4081 aggttgtgct ttaggtggcc taacgctggg acgcctgggc gatatttatg ggcgtaaaat
4141 cggcttaatg tgtgttatac tgggtgatgt tgttggtatc gtgatccaga ttgcttcctc
4201 tgacaaatgg tatcaatatt ttattggtag aattgtttct ggaatgggtg ttggagggtg
4261 tgctgtgctg tcgccaactt tgatctcaga aatttcccca aagcacctaa gaggcacttg
4321 tgtctctttt taccagctaa tgattaccct tggaaattttc ttgggctact gtaccaatta
4381 tggtagaaag aaatattcaa attcaatata gtggcgggtt cccttgggtt tgtgttttgc
4441 gtggggcaatc tttatgggtg ttggaattgg tatggttccg gaatcgccca gatatttagt
4501 agaaaaaggt aagtatgaag aagctagaag gtctttggcc aaatcaaaaa aggtcacagt
4561 tactgatcca ggcgttgttt ttgagtttga tactatagtt gcaaataatg aattagaaag
4621 ggctgttgga aatgccagtt ggcacgaact cttctcaaat aaaggagcaa ttctaccaag
4681 ggtaataatg ggaatcgtta tccagtcact gcaacagctt actggctgta attatttttt
4741 ctactacggc acgaccattt tcaatgctgt tggaaatgcaa gactctttcg agacttccat
4801 tgtccttggg gctgttaatt ttgcttctac atttgttgca ctatacattg tggataaatt
4861 tgggcgtcga aaatgtttat tgtgggggtc tgcctcgatg gcaatttgtt tcgtcatatt
4921 cgccaccgtt ggcgtcacta gattatggcc acaagggaaa gaccaacctt ctcgcaaaag
4981 tgctggtaat gttatgatcg ttttactttg tttcttcatt ttctcttttg ccattacttg
5041 ggctcctatg gcctatgtca ttgtggcaga aacttatcca ttaagagtta aaaatcgtgc
5101 catggccatt gcggttgggt cgaactggat gtgggggttc ttgattggat ttttcacacc
5161 ctttatcact agatccatag gattttctta tggctatgtt ttcattgggt gcttaatctt
5221 ttcgtacttc tacgttttct tctttgtttg cgaaacaaag ggattaactc tggagggaag
5281 taatgaaatg tacgaagaaa gaataaagcc atggaagtcc ggaggttggg tcccagttc
5341 tagaagaaca ccacaaccaa caagcagtag accattagtt attgttgata gtaataaatt
5401 tctaaatatt cttgtactct ggtaaacaga aataacaaca gataatggat tgatgcttta
5461 cttctatttt atggagattg gttttatata atagccttta ttaattggcg cacaatttga
5521 aaaaaaaatt aaaaaaata agacggacac atttgcgacg ccggtgataa aatgcatata
5581 agtagtttat aagctagcta ctactcagaa ataatttcaa aaaaaaaaag aagtgggcac
5641 tttaaaatga agattagcta tagtaaatta ctgtagtata acaactacct gctatctttc
5701 tgaaaaaatc aggcaatgta aggtcaaaact tgtaccatcg acatatataa tgttttgaga
5761 tataagtaac tagagaccag tttatacagg atcttacctc tttttaccgt tatgaaagct
5821 ttattactgc gttgttagta aacacacata ttattttcgg gtagtcctgt cgatgttcca
5881 aggcttttgc tttatcattg tttccgcttc atcgggtggt ttagtttagt tatctgtatg
5941 tgaagtatgt ggataggtgc ttctattatt ggaacaaaac acctaaacg cgcactaggt
6001 tataggaaa ggtccattat tcaaagaacg gcttattgaa aagtatgttg taaagctcgg
6061 tcatatcgcg catagccacg ataaggcgtg gtgctgcttc atagattgag aggcgcagtc
6121 aatacacacac cataccggtg gtttgataaa tgatactatc attccggaag ctctctagta
6181 agctgtaggt ggcataacag gtatctatca tctagtaata gctattgatt tttttctcga
6241 ctatgctata tgtgttggtg tggttcactt agaagtgaag aagtagcaat aaattagaca
6301 gacagaagta attgaagcta cattcaacaa catggctaag taaaaaagcg tagaatatca
6361 tgatgggttt ttttatcttt taattgcttt ctatgtagta taatggaccg atctttgcaa
6421 gtatatatct gtatgtatcc atatttagat ggcagcaagc aatatagatt tgatgagctt
6481 atatcatttt atcgtccttg tccaaaaagt cttgataaca ttaaaagtca ctaccgtcaa
6541 atccatcatc aaatccgccc tcgaaccac cagcatcatc aaatccgccc ctcgaccac
6601 cagcatcatc accgtagtaa ttgttctcga caacgacagt gtctggtccg tcatagttgt
6661 ggtcgtcaaa tgcgtgttct agcatagctc cacctaacaa acccgacca acaccaagta
6721 aaccacccat catggcaccg ccgtgtccac tacctttact ggtgcttggc gcagttccat
6781 agtatgcttg ctgtggtgct gctgctggca taggggcttg ttgaggatag taacgctgct
6841 gttgagggtg ttgagggtac tgaggctggt gaggctgata gtaacgagggt tgctgtgctt

```

SUBSTITUTE SHEET (RULE 26)

```

6901 gggcccctgc ttgcacttgc ggtgttgatt gagaagaata aggtggagga gcctgggtctg
6961 cctgttgacg agaactcttc tcattgttaa cacctgggtg aggacctttg ggtcttggcc
7021 atgttggtcc ccttggtggt tcccactgag agctgttcgt agataaatct acataatacc
7081 aagtctggta ttcacatca aaaacagcct tccagccaga aggcacttga ggaggattac
7141 ttttactttg agccattact ttaaaatggt ttgggttttt ttatcgactc tctttagtg
7201 agtgataaaa gcaattcaga aagagtcctt tgattatcat gactaaagta aactttttct
7261 tccagctgcc ggctcgggtca ggtggacgaa acaaaaaagc ctggtgaaat aatactgaat
7321 acgttaaaac gtcacagcgc ttgtatatct tgtgagagaa aggctattac cactgttata
7381 tgaatataga ttgctcaaca aagcgtgtct ttttctgtgt gcttaaaata tagtgtcttt
7441 tttgggaaaa aaacgtttat ttacagtatt ctcttcttct tctcctcatt caacttgttg
7501 cggttcgaag aaaattacgc ataaagaatc aaagaaaggc aaaaattacg ctgtacgatg
7561 ggggtcgtttc ccttagctga gtttccatta cgtgatatcc ctggttcctta tagctaccgt
7621 gtgtctggcg gtatagcttc ctcaggtagt gttactgcgc ttgttactgc cgctggcact
7681 catcgaaact cgtccacggc taagacagtt gagacagaag acggcgaaag agatatcgat
7741 gagtatcaga ggaaaagagc agctggttct ggcgaaatcca ctctgaacg cagtgttttc
7801 aaaagggtaa aacatgataa tcacaaaacc ctccatccag ttaacttaca gaacaccggt
7861 gcagcgtctg tggataatga cggctctgcac aatttaacag atatatccaa cgatgcagaa
7921 aaacttttga tgtctgtgga tgatggttct gccgcacctt ctacattgag tgtaaacatg
7981 ggagtggcat ctcataatgt tgctgtctcc actaccgtca atgctggcaac aataactggc
8041 agtgatgtta gtaacaatgt taatagtgtc actattaaca atcctatgga ggaaggagcg
8101 ctgccgttat caccactgc ttctctcca ggtaccacaa ctctttagc taaaactacg
8161 aaaactatca acaacaataa taatatcgcc gatttgatag aatccaaaaga ttctataatc
8221 tcccctgaat acctttctga tgagattttc agcgcaataa acaataatct cctcacgca
8281 tacttcaaaa atttattatt tagattagtt gccaacatgg ataggagtga actatccgac
8341 ttggggactt taatcaagga taatttaaag agggacctaa taacgtcttt gccttttgaa
8401 ataagtttga aaattttcaa ttatttgcaa ttcgaggata ttataaattc ccttggggtc
8461 tcccaaaaatt ggaacaaaat aattagaaaa tctacatcgt tgtggaaaaa acttctgata
8521 tcggaaaaatt ttgtgagccc aaagggtttt aattctctca atctcaaact ctccaaaaa
8581 tacccaaaac tctcacaaac agatcgctt agattatctt ttctggagaa tatattcatt
8641 ttaaaaaaatt ggtacaatcc caagtttgta ccacaaagga ccacgttaag aggccatatg
8701 acgagtgtta ttacgtgctt gcaatttgaa gataattatg tcattacggg ggctgatgac
8761 aaaatgatca gagtttatga ttcgataaac aagaaatttc ttctacaact atcagggtcat
8821 gatggtgggg tttgggcatt gaagtatgcc catggcggtg ttttagtcag cggttctaca
8881 gacagaacgg tgcgagtttg ggatattaag aaagggtgtt gtacccatgt gtttaaagggt
8941 tactctagaa cggtgagggt cctagatata gtagaatata aaaatatcaa gtacattgtt
9001 actggttcga gagataaac tttgcacgtt tggaaattgc ccaaggagtc ctccgttctt
9061 gatcatgggg aagaacatga ttatccatta gtctttcata cccctgagga gaaccatat
9121 tttgttggtg ttttaagagg acatatggca tctgtaagaa ctgtctcagg ccacggtaat
9181 attgtcgtta gtggctccta tgataatata ctgatttgtt gggatgttgc gcaaataaaa
9241 tgtttgtata ttttaagtgg acatacggat cgtatttatt cgacaatcta cgatcatgaa
9301 agaaaaaggt gcatctctgc cagtatggat accactatta gaatttggga tttggaaaat
9361 atatggaata atggagaatg ttctacgca acaaatcag catcgccatg cgccaaaata
9421 cttggtgcta tgtacacttt gcagggtcat acagcttttg tcggtttatt aagattatcc
9481 gacaaaattt ttggtcagtc cgctgcagac ggttcaataa ggggttggga cgcaaacgac
9541 tactctagaa aattttccta ccatcatacc aatttgagtg caattaccac attttatgta
9601 tcggataata ttttggtgag tggatcgga aatcagttca acatctataa tctacggagt
9661 gggaaatttg tccacgcaaa tattctaaaa gatgctgatc agatttgggtc gggttaatttt
9721 aaggggcaaaa cacttggtgc agcagttgaa aaagatggac agagcttttt agaaattctg
9781 gatttcagca aagcttcaaa aattaactac gttagcaatc ccgtaaaactc ctctcgtcgc
9841 tctttggaat ccatttctac ttctttgggt ctaacgagga caactataat accatgacct
9901 ttcccagaga ataagcattg actcatactt agataatata gcttaataag tagttatata
9961 atcagtaaaa aagtacaata acaacttcgt acattttatt gagtataaac tgcagctaaa
10021 ctgcctggat gtgtcaattt taatttgtgt taaaaaaagg gtgccgttta ttaattaatg
10081 tttcttccct gaaaatatgg aaagtacaag ttttagttg agaagggttt aagaaggtt
10141 tgaaaatgat ctaaaaaaat ataaaagcaa tcaaaagaaat aaaagctgga aaaatgcgta
10201 ataaccgaag tgactaaaat ttctttacgc gccaaataag aaatcgatgc tcttgaaagt
10261 agcaaccatt ttttaataaa tatattcctg atggttcttg gcccaagagt tttcttgaac
10321 ttttaacggt aagaagttga ttctgtgat ttttttcaag ttatcaagcg ttatgttttg
10381 aaacatctgt tccgttttca gtttttcaaa aagatcgata ttctcttgag aattaggaag
10441 ttctacgctc aacctccctg gggcagaaga aatacaaaag ctaatacaat tgtgttagaa

```

SUBSTITUTE SHEET (RULE 26)

10501 taaagtttcta atattatcta attagtagta ttcattgttac tagtatatta tcacatgata  
 10561 ttttctcaaat tggacaatgt aaataatgag tgttttttatg acacaatctt tatcgttaga  
 10621 tgttttcaacg ttccaaggct tgggttctatc gctcttctct tcaaattgta atcgtttgtc  
 10681 atgataaaca cgtacgggaa aaaaaaaaaa agtatcaata acgcgtaaag tgaatagagt  
 10741 attggattct ataagaccga agacgctcat atcacatctc ataaaatcac ttaaagcaag  
 10801 catccagagg ctattgataa aaagcaggca caaggagacg caatgggacg tttagttggc  
 10861 ttagaactaa gtaatttcaa gtcctataga ggcgttacca aggtaggatt cggcgagtgc  
 10921 aatttcacaa gtattatcgg tcctaacgga tctggtaaat cgaatatgat ggatgctatc  
 10981 tcattcgtac tcgggtgtgcg gagtaatcat ttgaggtcaa acatcttgaa agatttaatc  
 11041 tatagagggtg ttctaaacga tgagaatagt gacgattatg ataacgaggg cgtgcctct  
 11101 tcgaacccac aatccgcata cgtgaaggca ttttatcaaa aaggtaacaa actggtggag  
 11161 ctgatgagga taatttccag aaacggtgac actagttata aaattgatgg aaaaactgtc  
 11221 tcctataagg actattctat atttcttgag aacgaaaata ttcttatcaa agccaaaaat  
 11281 tttctagtgt tccaggggtga tgttgagcaa attgcagcac aatctcccgt agaattatca  
 11341 agaattgtttg aagaagtatc aggttctatc caatacaaaa aggagtatga agagttgaag  
 11401 gaaaagattg agaaattaag caaatctgca accgaatcta taaaaatatg gagaaggatc  
 11461 catggagaat tgaagacata taaagaaggt atcaataaga acgaggagta taggaaacaa  
 11521 ttggacaaaa aaaatgaatt acagaagttc caggctctat ggcagttata tcatttagag  
 11581 caacaaaaag aggagctaac ggacaagctg tccgcattaa actctgaaat atcgtcttta  
 11641 aagggaaaaa taaataacga gatgaaatca ttacaacgct caaaatcttc ctttgttaaa  
 11701 gaaagcgcag taatttctaa gcaaaaaagt aaatttagatt atatcttcaa ggataaggaa  
 11761 aaatttagtct cggatttacc gctcataaag gttcctcaac aggagcagg gaagcgaatt  
 11821 tcccatatag aaaaaagaat cgaaagttta cagaaagatc ttcaaagaca gaagacttat  
 11881 gtggagagat ttgaaacaca actaaaagtg gtgaccagat caaaggaagc ttttgaagag  
 11941 gaaatcaaac aatctgctag aaactatgac aaattcaagc taaatgaaaa tgattttaaag  
 12001 acatataatt gcttacatga aaaatatctg actgaagggtg ggtcaatcct agaagaaaaa  
 12061 attgccgttt tgaacaacga taagcgagaa atccaagagg aattggagag attcaacaaa  
 12121 agggcagata tttctaaaaa aaggataacg gaggagcttt ctataacagg agaaaaattg  
 12181 gacacgcaat taaacgattt aagagtttct ttgaatgaga aaaacgcctc tcatactgaa  
 12241 cgttttgcacg agctgaaaaa attacaatct gatattgaat ctgctaataa tcaagaatac  
 12301 gactttaatt tcaagttgag ggaaacggtg gttaagatcg atgacttgag tgctaataca  
 12361 agagaaacaa tgaaagaaag aaaactaaga gaaaatatag caatgttgaa aagattcttc  
 12421 cccggagtaa aaggtcttgt tcatgatctt tgtcacccaa aaaaggagaa atatggcttg  
 12481 gcagtgtcta ccatcttagg taagaacttt gattccgtca ttgtagaaaa tttaccgta  
 12541 gctcaagaat gcattgcatt tttgaagaag caacgtgcgg gcactgcac tttcatacca  
 12601 ctagacacaa ttgagacaga gttacctaca ttatcattgc ctgactcaca agactatatt  
 12661 ttatcaatta atgctattga ctacgagccg gaatatgaaa aagcgatgca atattgtgtg  
 12721 ggcgattcca tcatatgtaa tacgttgaat attgtctaaag atctgaaatg gaaaaagggc  
 12781 ataaggagga aattggttac aattggaagt gctttgatcc acaaggccgg tttgatgaca  
 12841 ggtggtatat caggagatgc caataatagg tgggataaag aagaatatca aagcttaatg  
 12901 tcttttaaaag acaaattact aatccaaatc gatgaacttt ccaacgggtca acgctcta  
 12961 tcaatcagag caagagaagt tgaaaatagt gtttctactat tgaactcgga catagcaaat  
 13021 ttgagaactc aagtaacaca acaaaaacgc tccttggtatg aaaatcggtt agagattaag  
 13081 taccataatg acttgataga gaaagaaatt caaccgaaga taactgaact aaagaagaag  
 13141 ctagatgatt tagaaaatac taaagataat ttagtgaaaag agaaggaggc tttacaaaat  
 13201 aatattttca aagaattcac tagtaaaatt ggcttttcaa tcaaagaata tgaaaatcat  
 13261 tccggtgaat tgatgagaca acaatctaaa gaattacagc agttacaaaa acaaattttg  
 13321 accgttgaaa ataagttgca gtttgagaca gacagactaa gtactactca aagaagatat  
 13381 gaaaaggcgc aaaaggattt agagaatgct caagttgaaa tgaagtcttt ggaagaacag  
 13441 gaatatgcaa tagaaatgaa aatcggatca atagagtcta aattggaaga acacaaaaat  
 13501 cacttagatg agttacagaa gaaatttgta acgaagcaaa gtgaattaaa ttccagcgaa  
 13561 gatattctag aggacatgaa cagcaactta caagtcttaa aaagggaag agacggtata  
 13621 aaggaagata ttgaaaagtt tgatttagag agagtaacag cgttaaagaa ttgtaaaatt  
 13681 tctaataata atatacctat atcatctgaa acaacgatag atgatttacc aatatcttc  
 13741 actgataatg aagcaattac aatttccaac agtatcgata taaactataa aggactacct  
 13801 aaaaaatata aagaaaacaa taccgattcg gcaagggaag agctggagca gaagattcat  
 13861 gaagtggagg aaatattgaa cgagttgcag cccaatgcaa gagctttgga gagatacgac  
 13921 gaggcggaag gaagggttga agtgattaat aacgaaacag aacaactaaa ggccgaagaa  
 13981 aagaaaatat taaaccagtt cctaaaaatt aagaaaaaaa gaaaggaact gttcgaaaag  
 14041 acatttgatt atgtgagcga ccatttagac gcaatctaca gggaacttac taaaaatccc

SUBSTITUTE SHEET (RULE 26)

14101 aactccaatg tgggaattggc cgggtggtaac gcttcttttaa ccatagaaga cgaagatgaa  
14161 ccgttccaatg cgggaatcaa atatcatgcc actccgcctc ttaaaagatt caaagacatg  
14221 gaatatcttt ctgggtgggtga aaaaaccgta gctgcattag ctctattatt tgctattaat  
14281 tcctaccagc ctagtccctt ctctcgtgctg gacgaagtgg acgcagccct agacattact  
14341 aacgtccaga gaattgctgc ctatataaga aggcaccgta atccagatct ccaattcatt  
14401 gtcatttcat tgaagaacac catgtttgaa aaatctgacg ctctcgttgg ggtttacaga  
14461 cagcaacaag aaaactcgtc gaagatcata actttggact tgagcaatta cgcagaataa  
14521 tctatgaaac caacctctgc tataaccctg caaataacta ataatatcta tataggtcaa  
14581 ctagctagtg caatatcata gtaacaataa tattaataac gtcacttttt ttccagggtgta  
14641 acccaattgt ggtgggtggc ggccgaggta tcccttagaa aagaattttt taagtctctt  
14701 ctcatctctt accagtggag aagtacacga tatttgcaaa gtctgtcatc agggccttgat  
14761 aataaagctg cattagatct tagcaaaaac tacgagaaga acattgaata ttgtagctgt  
14821 atttgcatac ataaacttta tcatgttctg ttagctagct ttgcacatta atttttcgat  
14881 ttgttaccgc caatgaccgc taacaatgac gatgatata aatcacccat tcccattact  
14941 aacaagacct tatcccaatt gaagcgcttt gagagaagtc caggaaggcc cagttcttct  
15001 cagggcgaga taaaacgtaa aaagtctagg ctatatgccg cagacggaag accacattct  
15061 ccgctaagag caaggtctgc taccccaacg ctacaggacc aaaaactggt caatggcatg  
15121 gattccactt cctttttgaa tgaaaggcta cagcattata cgctggatta tgttagtgac  
15181 agggcccagc atatgaagaa tatatatgac ccatcatctc gctgggttcag cagatccgtg  
15241 aggcctgaat ttcttattga agagttctta ccatataaga ctgaaagtca tgaagatcaa  
15301 gcgaaatact tgtgccatgt cttagttaat ctttatattg cgatcagctc attagatata  
15361 caaggtttga tttctatttc cagtaaaagat ctggctgatt taaagaaaga agtggatgat  
15421 ttagctctta aaaccgatct tttcaggtta tctaacaaca cagcggagaa tgacttactt  
15481 ggtaacgata ttgctgatta tgacgatgcg gaaggcctgg aggacgaatt ggatgaatac  
15541 ttcgacttag caggccccga ctttaatgct accgaaaaa tcacagctaa atcagctact  
15601 attgtgaatg taaaccattg gactaatgag ctcaaaaatt gtctacattt tgattttcca  
15661 gtagctttta gaaagtcgct agcgacagtt tattattatt tgtctcttgt tcagggccaa  
15721 aaggtgtata gacaaatgca tgtcgatatg ttcgaaagat tagtaagcct tgacgatgat  
15781 aggacaaatt tcaactgaact gttgcaaaaa caaggtcttt tgctagatca tcaaatcatg  
15841 ctcaattttcc tgtgcgaatt tctacccttat ccagatcccg actatgctcg ttatgaatta  
15901 tcatcaaagg aagattttaca gttattttaga ttacttttga agcatgcaca taatgcaaaa  
15961 ccattttttcg ataagtcaaa ggaaagtttg ttagttgata cgatgaattt tctgttgtct  
16021 agtcttgcac catctactat gatggctgtc atgcctatcg ttacatccgt tgtgccctat  
16081 cattatcata tccattctaa gatcatcgat tatttcccgt tttgctatag catctggagc  
16141 tcagtcagcg caaacgtggc catcgacact cacatgtatg attttgttgg gtcaattttcc  
16201 aaagacgttc ataataagat tttgagtagc gagcatgaaa aggatgtagt tgggtagag  
16261 tttggagaat tcgggatttt tactgatgat caaatgactt ttatgttcaa taggttacaa  
16321 ggccatctta gaacagacgg tcaaatacat tcgtattccc gcacagtga gctttttgtt  
16381 tatgctataa acggatcaaa aaaagatagg ttttttgaaa aacttgtaag tttagccaaa  
16441 gcaatcgaaa catttatcca tccctctaatt aatgggtttt ggaccaagcc aaatgctaaa  
16501 ttcgttcatg catttataaa gtcttaccac ggaaggggtca aatatgaaga agatatttgt  
16561 gctagggggcgc tcacaaatgg gatattgttta acttcttttt gtcacgaaga gatagttgaa  
16621 atattcttaa atattatcag tctgggttca cagaataaaa atcctgatat tgcgaactat  
16681 tacatctctt gtttcgcata tctgttagag ctggatcctt caaatgcata ttttaattat  
16741 gacaaaatac tgatagattt gtacgatacg ctggctgacc aatttatcaa ttcgagacac  
16801 agaatcattt cctctttgaa acaattttaca agagtaattc gggtttattgt gatggataag  
16861 ctatatcgcg tgcacataac aaacgtcctt tcgatgctgg tctccaaact tgatatgaac  
16921 gatactaatt tgacaagcaa cctcatcaac ggtattgtat ctatagccgc tttcattcct  
16981 atccaagacc tcaactgggga ggacgattat atatcgtttg aatcggatac tcttccttta  
17041 gttcaacaac atttttatca tattaagtgt ggcgaaagt tccactacag tttttcaaag tatgctaaaa  
17101 gatgaactgt taaataacgc tttcaaagct gatgtagact tagaggactc tttggttact  
17161 gtatacgtag aaaaaatttt ccaattgggt agtctatgg acgataaaa atttaattat  
17221 aaaaataacc aaacaactat gattttacaa agtaatgact ccttcaagga aaggatcca  
17281 tttgcttctt tattacagag aaacttctgg gctttagtaa gaaggaataa tgggtttgagt  
17341 aactatgaat tagttactat ccactagcg gctttagtaa gaaggaataa tgggtttgagt  
17401 aaagagttgg tcagaactct tttattccat atcaaagaac aaatcaaaag aggcgccggg  
17461 tctgtaagaa gtacttcaga gattcaacag agggatgtta agttagtttt atacttaact  
17521 gactaaatg atgtcttaag gcaatgtcat gaatctctat tggagtatag cgatgagttg  
17581 ataacattca tgaaatattt atacgacaac gtcactaacc cgccattgga tgttatcaca  
17641 tctattgtta ttcacagtgc tttagcaact ctatgtacaa ccgaaataac tgattgtcgt

## SUBSTITUTE SHEET (RULE 26)

6/21

```

17701 ctattccag aggactctaa gattccggaa aaagacagat ggggaggact acagttcgat
17761 cctcgaagat ttgataaaca gcatttgagt tttcagtggc acgtaccttc tagtgacgag
17821 ataactttat ccataagcat tctagaaagc ctttccgaat actgtattaa caacgtagaa
17881 gaactgatga aagctccaag acatgattcc gaatatggcg atatgatata aaaatatgtt
17941 ttagttatga cacatacgct ttccgggtca agtttacttt ttgatccaga ttttaacaaa
18001 tataggacgc aatcaaactt atcatacaga gagaaactga ttttattaaa gaatatacgc
18061 gaaaataatt gtgaccctca agaactggat attgatattg aacaaattcg ttctggcaag
18121 gatgatgaag actatattga gagtaaggac attgaagcag ggctgaatgc aggagtttcc
18181 gatggtgtgc agttaagaga tgagtttccg gatgaattaa ttggtgatga agaagtagtg
18241 tctgagatgc catctggtgt aaataccctt atcgcgggga cgcattggcac ggacaattca
18301 gctatgagtt cggatctagc tttcagggat ttagatattt acacctgtaa ttattacttc
18361 ggaaataacca ctgaggagaa gttacaaaac ccacaatatt tacaagtcca cagagttaga
18421 gcgcgcattg gacatttctt tcacaaactc tatgtttttt tatctacaaa cttcgaaaac
18481 aacaccaaca tgttccaaat tctattgcac ggattgaaag tttggttcac agatctggga
18541 caagaaacgg tcttcaatga agacccaaat gccttcattg acgttgattt cctagaaaac
18601 gttcaatctc tctcacacgt aaatgagccc ttcacgagaa ccaattttgc aatcagagca
18661 aacagtttgc accagagtag agttctatta cattcaacaa atagaaaagc ttccaagctg
18721 gaaaacctat tgtagttga catcatacag tttagcagat ccctttatct tgatatttat
18781 aaaccagcac agggaaactt ggtacactgt atgaaacaat tagttgggtc atatggcgta
18841 gtcacataa aaattattcc atcatttagag aaagcgatta aggatcatga ttatatgaaa
18901 atccaagtta ttttaaattg tttgttaatt aagaaaatcc ataggaagct tatgacggat
18961 tataaagaca tcggcagatt gatatttctg cttattgaat gttgtcgtgt gaatgaatta
19021 gagattggta tgtatgcaga taaaatctta actgacatag tgattgggat caagattcct
19081 tctagtgtat gtgtcatttc cgatcaagct ttcttacctt tagcacctcc tgatgggtact
19141 attaatttgc aagttgaagc ggtaaagctt gccaaaaaga aaaagcgtga gtactacctc
19201 tctctgttag tggatttgca ggacaaactt ttagacaaat tagataatga aaaagatatg
19261 gggtggaaga taagaatgtt cattttacgt tttgttacac aaatccaatc gaacctcgaa
19321 agcaaaccg ataaaagagc agtattttca ataactctcc aaatctccac aaaacatccc
19381 gaaatcatac atttggttgt aaagtcattg ttgtcgacgt gcaacaagat aatatctctc
19441 tctgactatg aatatgacat caccagggcc tataagaatg aattcaatcc atcatttgtg
19501 gaaatactgg acacttcgac cacaagcttc ctaaaaacgt ttactgaaga aatgaataac
19561 tttgataacc ccaagtattt tatcgatttg agggcgtag taggggtggc atgttgggga
19621 aggcttatgt acgtcatgtc gccgaaagct ttaaagctca atttacgtga gaatgaactg
19681 gaagtcctca agacagctgg tcatctattg acaagagaat tcctgagaga tgttacaatg
19741 aatttagtcc aagataatga aactaggggt gtttttagta gtggtaacgt gtcatttttc
19801 tctttagtaa tccttttgat atcatctggt ttctgcgaac tgaatatgtc ggatctctt
19861 gagctatgtg aatcctacta taacaaagac gataaggctt cgatgatcat gtctgtcgag
19921 atagtggtg gcttagtttg cgggagtaag tttatgtcag tctctgactt ggacaaacgt
19981 gacactttta tcgaaaactt cctagccaaa tgttttagatt atgagttgaa ccatgacgca
20041 tttgaaattt ggagcacctt ggcattggtg ttgctgcag tcggtgattt aagaaggtct
20101 aaaacttttt tttgccattt tatcaacgcc gatggcatgt ttgaccgtga atctgatgca
20161 gccacacatc aaacctccaa aatttacatg ctaagaagta tcttgatgag catggaattt
20221 agagccccag atgttggtta gctatttgat gagttgggtat ttgatcacc atacgatcag
20281 gttegccagc tgtecgtaaa ctattgacga ccttagttca aaatcaaagt aatccgtcaa
20341 tttcagatcc aaccacatta ttagaagcag aacggaatga tcc

```

SEQ ID No. 2

```

1 mgsfplaefp lrdipvpysy rvsoggiassg svtalvtaag thrnsstakt vetedgeedi
61 deyqrkraag sgestpersd fkrvkhndnhk tlhpnvlqnt gaasvdndgl hnltidisnda
121 eklmsvddg saapstlsvn mgvashnvaa pttvnaatit gsdvsnnvns atinnpmeeg
181 alplsptass pgtttplakt tktinnnnni adlieskdsi ispeylsdei fsainnnlph
241 ayfknllfrl vanmdrsels dlgtlikdnl krdlitslpf eislkifnyl qfediinslg
301 vsqnwnkiir kstslwkkll isenfvs pkg fnslnklslq kypklsqqdr lrlsflenif
361 ilknwynpkf vpgtrttlrgh mtsvitclqf ednyvitgad dkmirvydsi nkkfllqlsg
421 hdggvwalky ahggilvsgs tdrtvrvwdi kkgccthvfk ghnstvrcl d ivelykniky i
481 vtgsrdntlh vwklpkessv pdhgeehdyp lvfhtpeenp yfvgvlrghm asvrtvsghg
541 nivvsgsydn tliwvdvaqm kclyilsght driystiydh erkrcisasm dttiriwdle
601 niwnngecsy atnsaspcak ilgamytlqg htalvgllrl sdkflvsaaa dgsirgwdan
661 dysrkfsyhh tnlsaittfy vsdnilvsgs enqfniynlr sgklvhanil kdadqiwsvn

```

## SUBSTITUTE SHEET (RULE 26)

7/21

721 fkgkctlvaav ekdgqsflei ldfskaskin yvsnpvnsss sslesistsl gltrttiip

SEQ ID No.3

```

1  tgcgttggtc  gcggcctggc  accaaagggg  cggccccggc  ggagagcgga  cccagtggcc
61  tggcgatta  tggacccggc  cgaggcggg  ctgcaagaga  aggcactcaa  gtttatgaat
121  tcctcagaga  gagaagactg  taataatggc  gaacccccta  ggaagataat  accagagaag
181  aattcactta  gacagacata  caacagctgt  gccagactct  gcttaaacca  agaaacagta
241  tgttttagca  gcaactgctat  gaagactgag  aattgtgtgg  ccaaaacaaa  acttgccaat
301  ggcacttcca  gtatgattgt  gcccaagcaa  cggaaactct  cagcaagcta  tgaaaaggaa
361  aaggaactgt  gtgtcaaata  ctttgagcag  tggtcagagt  cagatcaagt  ggaatttgtg
421  gaacatctta  tatcccaaact  gtgtcattac  caacatgggc  acataaactc  gtatcttaaa
481  cctatgttgc  agagagattt  cataactgct  ctgccagctc  ggggattgga  tcatatcgct
541  gagaacattc  tgtcatacct  ggatgccaaa  tcactatgtg  ctgctgaact  tgtgtgcaag
601  gaatggtacc  gagtgcctc  tgatggcatg  ctgtggaaga  agcttatcga  gagaatggtc
661  aggacagatt  ctctgtggag  aggcctggca  gaacgaagag  gatggggaca  gtatttatc
721  aaaaacaaac  ctctgacgg  gaatgctcct  cccaactctt  tttatagagc  actttatcct
781  aaaattatac  aagacattga  gacaatagaa  tctaattgga  gatgtggaag  acatagttaa
841  cagagaattc  actgccgaag  tgaaacaagc  aaaggagttt  actgtttaca  gtatgatgat
901  cagaaaatag  taagcggcct  tcgagacaac  acaatcaaga  tctgggataa  aaacacattg
961  gaatgcaagc  gaattctcac  aggcataaca  ggttcagtc  tctgtctcca  gtatgatgat
1021  agagtgatca  taacaggatc  atcggattcc  acggtcagag  tgtgggatgt  aaatacaggt
1081  gaaatgctaa  acacgttgat  tcaccattgt  gaagcagttc  tgcacttgcg  tttcaataat
1141  ggcagtatgg  tgacctgctc  caaagatcgt  tccattgctg  tatgggatat  ggccctccca
1201  actgacatta  cctccggag  ggtgctggtc  ggacaccgag  ctgctgtcaa  tgtttagtag
1261  tttgatgaca  agtacattgt  ttctgcatct  ggggatagaa  ctataaaggt  atggaacaca
1321  agtacttggt  aatttgtaag  gaccttaaat  ggacacaaac  gaggcattgc  ctggttgtag
1381  tacagggaca  ggctggtagt  gagggtccta  tctgacaaca  ctatcagatt  atgggacata
1441  gaatgtggtg  catgtttacg  agtggttaga  ggccatgagg  aattggtgag  ttgtattcga
1501  tttgataaca  agaggatagt  cagtggggcc  tatgatggaa  aaataaagc  gtgggatctt
1561  gtggctgctt  tggacccccg  tgctcctgca  gggacactct  gtctacggag  ccttggtagg
1621  cattccggaa  gaggttttcg  actacagttt  gatgaattcc  agattgtcag  tagttcacat
1681  gatgacacaa  tcctcatctg  ggacttccta  aatgatccag  ctgcccagc  tgaaccccc
1741  cgttccccct  ctggaacata  cacctacatc  tccagataaa  taaccataca  ctgacctcat
1801  acttgcccag  gacccattaa  agttgcggt  tttacagtat  ctgccaatac  caggatgagc
1861  aacaacagta  acaatcaaac  tactgcccag  tttccctgga  ctagccgagg  agcagggtt
1921  tgagactcct  gttgggacac  agttggtctg  cagtcggccc  aggacggtct  actcagcaca
1981  actgactgct  tcagtgtctg  tatcagaaga  tgtcttctat  caattgtgaa  tgattggaac
2041  ttttaaacct  cccctcctct  cctcctttca  cctctgcacc  tagttttttc  ccattgggtc
2101  cagacaaagg  tgacttataa  atatatttag  tgttttgcca  gaaaaaaaaa  a

```

SEQ ID No. 4

```

1  mdpaeavlqe  kalkfmsns  redcnngepp  rkiipekns  rqtynscarl  clnqetvcla
61  stamktencv  aktklangts  smivpkqrkl  sasyekel  cvkyfeqwe  sdqvefvehl
121  isqmchyqhg  hinsylkpl  qrdftalpa  rgldhiaeni  lsyldakslc  aaelvckewy
181  rvtsdgmlwk  kliermvrt  slwrglaerr  gwgyllfknk  ppgdnappns  fyralypkii
241  qdietiesnw  rcgrhslqri  hcrsetskgv  yclqyddqki  vsglrdntik  iwdkntleck
301  riltghtgsv  lclqydervi  itgssdstvr  vwdvntgeml  ntlihhceav  lhlrfnngmm
361  vtcskdrsia  vwdmasptdi  tlrrvlvghr  aavnvdfdd  kyivsagdr  tikvwnstc
421  efvrtlnghk  rgiaclyrd  rlvvsgssdn  tirlwdiecg  aclrvleghe  elvrcirfdn
481  krivsgaydg  kikvwdlva  ldprapagtl  clrtlvehsg  rvfrlqfdef  qivssshddt
541  iliwdfldp  aaqaeprrsp  srtytyisr

```

SEQ ID No. 5

```

1  cacgctaate  atgactcaaa  taaatccata  aagttttata  cagtttttaa  aaatatcatc
61  atctattacc  cagatgtgtt  aatgaaccat  tctatagtca  tcattactag  gctttcatta
121  ctactgaggt  taccgccta  tggaccttcc  ttggtaaagg  aacttgtttt  aaaaattggc
181  ttttaacaaa  tttagtata  tgattatcaa  aaaaggcgtg  gcaaaatata  aaacacaaa
241  ttttaactgt  cctgtgtgtt  actttctttt  gtccatactt  caccagtttt  tcgattttac

```

SUBSTITUTE SHEET (RULE 26)

```

301 acaataattc gttttcattt aatcgttctc ttagaagccc ggtttttgaa tatcaaaatc
361 gtacttggtg ccaactagca gggaagccca aaaattaagg cattgcattt aagcttacac
421 ctgctgtaaa tcttgaaatt tctcattgat ttcggcacaa taattatcat tggtagtgag
481 gctaaacagt ttgcgggtttc ctttatacta agaaggtcta taatggatca ggataacaac
541 aaccacaatg acagcaatag gctgcaccca cctgatatac atccaaatctt gggccctcaa
601 ttgtggctga atagtagcgg tgattttgac gacaacaaca acaacaaca caacaacaac
661 aataataata gcacaagacc acaaatgcc aacgaacta gagaaacggc aacttcggaa
721 agaaatgcaa gtgagggttag ggatgcaacg ctaaataata tctttagggt cgatagtatt
781 caacgggaaa cgcttttgcc aaccaacaac ggacaaccgc taaatcaaaa cttttcgctg
841 acatttcaac cacaacagca aacaaatgcg ctgaacggga ttgacataaa cactgtgaac
901 acaaacctta tgaatgggtg caatgttcaa atagatcaac ttaatcgatt gttaccgaac
961 ctaccagagg aagaacggaa gcaaattcac gaattcaagc taatagtggg caaaaaaatc
1021 caagagtttc tgggtgttat agagaaacgt agaaaaaaa tactgaacga aattgagcta
1081 gacaacctta aactaaagga gctacgtatt gataactccc cacaagcaat tagttatttg
1141 cataaattac aaagaatgag gcttagggcg ctagagacag aaaacatgga aattagaaat
1201 ttaaggctaa aaatattaac aattatagaa gagtacaaaa agtcattata tgcatactgc
1261 cattccaagc taagagggtca acaagtggaa aatccaacag ataatttcac catttgata
1321 aactccatag atactactga atcatctgac ttgaaagaag ggctacaaga tctttcgagc
1381 tattcaaggc agttcataaa taatgtgctt tccaacatgc caaatcaaaa catatgtacg
1441 agtgtcacc gaagatcacc tgtgtttgcc ctaaacatgc taccctcgga aatattacac
1501 ttaattattg ataaacttaa ccaaaaatat gatattgtaa aattccttac cgtttccaaa
1561 ctctgggctg aaataattgt gaagatactt tattacagac cgcacatcaa caaaaagagt
1621 caattagact tgtttttaag gactatgaag ttaacttctg aagaaactgt attcaactat
1681 cgtttaaatg tcaaaagatt aaatttttca tttgttggtg actacatgca cgatacagag
1741 cttaactatt ttgtcggatg taagaatttg gagcgactaa ctttagtatt ttgcaagcat
1801 ataaccagtg ttccaatatc ggctgttttg agaggatgta aatttctcca aagtgtggat
1861 atcactggaa taagagacgt ttccgatgac gtatttgata ccttagcgac atattgtccc
1921 agagtacagg gcttttatgt tcctcaggca aggaatgtaa cattcgattc actgcggaat
1981 ttcatagtcc attccccgat gttgaaaaga ataaaaatca cagcaaaaaa taacatgaat
2041 gacgaattag tagaactatt agccaacaaa tgccctttgc ttgtagaggt cgatataaca
2101 ttaagtccaa atgtcactga ttctagtttg ttaaaactcc tcactagggt agttcagctg
2161 aggggaattc gaataactca taatacgaat attacggata atcttttcca ggagctttct
2221 aaagtagttg acgatatgcc ctctttaaga ttgattgatc tttctggatg tgaaaaatatt
2281 acagataaaa ctatagaaag tatagtcaat ttagccctta aattacgtaa tgtttttcta
2341 ggcaagtgtg gccgaattac agatgcatcg ttgttccaat tatcgaagct gggcaaaaac
2401 ttgcaaacag tgcattttgg gcaactgttc aatataactg ataacggggt aagagcactc
2461 tttcattcat gtacaagaat acagtatgtg gactttgcgt gctgtacgaa ttaaccaat
2521 agaactcttt atgaactagc agacttacca aaattaaaga gaattggcct tgtcaaatgt
2581 acgcaaatga ctgacgaggg tttgttgaat atggtttctt tgcgaggccg aaatgatact
2641 ttagaaaggg tacatttatc ttactgttct aatttaacaa tatatccgat atatgagctt
2701 ctaatgtctt gcccaaggct ctacacattg tctttgactg ctgttccgct atttttacgc
2761 cccgatataa cgatgtattg caggcctgca ccctcagact ttagtgaaaa tcaacgtcaa
2821 atattctgcg tattttcagg gaaagggtgt cataaaactc gccattattt agtaaattta
2881 acgtcgcccc cttttggacc acatgtcgat gtaaattgat ttttgacaaa atatattaga
2941 tccaagaatt tgatatttaa cgggtgaaaca cttgaagatg ctcttaggag aatcataact
3001 gatttaaate aagattccgc tgcaattata gctgtacag gattaaatca aatcaacggg
3061 ctaataaacg attttctttt ccagaaatc aattttgaac gaatagatga agtattcagt
3121 tggatatctc atacttttga tggcattagg atgagctcgg aggaagttaa ctactatta
3181 ttgcaagtaa acaagacgtt ttgtgaagat ccatttagtg atgtggacga tgatcaagat
3241 tatgtcgtag cacctggtgt aaaccgggaa attaacagtg aaatgtgtca tattgttaga
3301 aaattccatg agttaaatga tcatattgat gatttcgagg tgaatgttgc tagtttggtg
3361 agagtccagt ttcagtttac tgggtttttt cttcatgaaa tgactcaaac ctatattgaa
3421 atgattgaat taaacagaca aatttgttta gtacaaaaaa cggttcagga atcgggcaac
3481 atagattacc aaaaagggtt tttaatatgg cgacttttat tcattgacaa attcattatg
3541 gtggttcaga agtacaagct ctccaccgtt gttttgagac tatattttaa agataacata
3601 acattgttaa ccagacaaag agaactatta atagcccacc aaagatcagc atggaataac
3661 aataatgaca atgacgccaa ccggaacgcc aacaacatag tgaatattgt atcggatgct
3721 ggggcaaacg atacaagtaa caatgaaact aacaatggta atgatgacaa tgaaacagaa
3781 aatccaaatt tctggcgta gtttggaat agaatgcaa tatcacctga ccagatgagg
3841 aatctccaaa tgggacttcg taatcagaac atggttagga acaataacaa caacacaatt

```

SUBSTITUTE SHEET (RULE 26)



9/21

```

3901 gacgaatcaa tgcctgacac tgccattgat tctcaaattg atgaagcatc aggaacgccc
3961 gatgaagata tggtataatt gtattttcatt gaataacttac tgtcctacta caccttttatt
4021 ttcaaaatcc cactttttctt acttattttac atataaatac ataatgcatt cacttttgaaa
4081 cttttttgcct tagcatgtat acgctataga cttgcggtat caacgaatat acgtaacggt
4141 gtcacgtcca cagaagatgc tatgtcaaca aaatacaaac ctttagcctg tttatcaaat
4201 acattctata cacagtttca aaactacaaa aaatacaaac ctttagcctg tttatcaaat
4261 tagtttagcta taaaatgccc atttttcttag caatatcgat caattgattg tcatcttcca
4321 aagtttcaat aaaattttgtg gcagtatagt aatcccttct caatatgtcc aattttcttat
4381 cacagtcac tataaattcg cgcttaatat attgcacttt taggttcacc caattgttga
4441 atgtcatgat ccaactcttc tgcaggattt

```

SEQ ID No. 6

```

1 mdqdnnnhnd snrlhppdih pnlgpqlwln ssgdfddnnn nnnnnnnnns trpqmpsrtr
61 etatsernas evrdatlnni frfdisigret llptnngqpl nqnfsltfqp qqqtnalngi
121 dintvntnlm ngvvnqidql nrllpnlpee erkqihefkl ivgkkiqefl vviekrrkki
181 lneiieldnlk lkelridnsp gaisylhklq rmrlralete nmeirnlrlk iltiieeykk
241 slyaychskl rgqgvenptd nfiiwinsid tressdlkeg lqdlrsysrq finnvlsnps
301 nqnictsvtr rspvfalnmrl pseihlild klnqkydivk fltvsklwae iivkilyypr
361 hinkksqldl flrtmklts etvfnyrlmi krlnfsfvgd ymhdtnlyaf yvcknlerlt
421 lvfckhitsv pisavlrgek flqsvditgi rdvsddvfdt latycprvqg fyvppqarnvt
481 fdslrnfivh spmlkrikit annnmndelv ellankcp111 vevditlspn vtdssllkl1
541 trlvqlrefr ithntnitdn lfgelskvvd dmpslrlidl sgcenitdkt iesivnlapk
601 lrvnflgkcs ritdaslfql sklgknlqtv hfghefnitd ngvralfhsc triqyvdfac
661 ctnltnrtly eladlpklkr iglvkctgmt degllnmvsl rgrndtlerv hlsycsnlti
721 ypiyellmsc prlshls1ta vpsflrpdit mycrpapsdf senqrqifcv fsgkgvhlkr
781 hylvnlts1a fgp1hvdvndv ltkyirsknl ifngetleda lrriitdlng dsaaiaaatg
841 lnqinglnnd flfqninfer idevfswyln tfdgirmsse evnslllqvn ktfcgedpfsd
901 vdddqdyvva pgvnreinse mchivrkfhe lndhiddfev nvaslrvvqf qftgflhlhm
961 tqtymqmiel nrqiclvqkt vqesgnidyq kglliwrl1f idkfimvvqk yklstvv1rl
1021 ylkdnit1lt rqrrelliahq rsawnnnndn danrnanniv nivsdagand tsnnnetnngn
1081 ddnetenpnf wrqfgnrmqi spdqmrnlqm glrnqnmvrn nnnntidesm pdaidsqmd
1141 easgt1pdedm 1

```

SEQ ID No. 7

```

1 tttctt1gttg cttcaacgga ttatcttaaa aaaatctatc atattttcaaa atataaattc
61 ttattttttac aaagaagata tagattatgc ataataattat tttgtttacat tttttttctt
121 ttactttttta ttttcttttc tttgtacttc ctcaaataag catcttttagc aggttaacaa
181 tagcaaatat catgtccagc ccaaatcaca taatactttc cacttcactt tcaactatctg
241 tttcgggaga cctatcatcc acggtattga cagttgggtct ttggaaatca gttgcatgtg
301 ccaaggaat ggatctcgaa atgttctcaa acacggagga ccttcttctg aaagtccac
361 ggtaagcggg cataggttcg gcaaaactgt gaggattatt gtcattgtgaa ggcgtgatag
421 gtatgtcgtt aagtgatgaa aaggcttttg aaccctaaatc actactatcc atagaatcat
481 caccgacctg cgcaattgga taaggcagag gagcgtcacc ggtaaccctg aataggtgaa
541 cattggcaac atgttcattt tgcactctga agatgatcca aacgaatctt cttaaaacct
601 ccaaaagggc caataataaac gacgtcacag cactctgctg aattgtttga ggagcaattg
661 cgtatacaat ccactcaaat ctaatgagga tatcccaaat catagcaaag taataaacta
721 acttcttact gaatgaataa ctgccgtttt cccaattttt tttaccagct aaatacaagt
781 cgtctcttag caaccaattg taagaagtag tattgtgagc aaaggaccaa tccataacta
841 aatcccaggc ggaagtaagg atcgaattca acgtagcgca caccgataaaa ggggttctcc
901 tttgttcaga acggtctgac aatctgtaag cacaaagtgt ggcattatac gctataccca
961 aagtgtattt cgccgcgttc aaaagatgag ggaaccaatc accggaatca gcaaatcttc
1021 gtaaacattg catgaatctc caataacttg gtaaacatga taaaacaccc attgctctgg
1081 aatgtgaaga gccacataga ttgttgggcg tatgagagta aacacagaag aacatggcaa
1141 tatcggcaat agaatacgtc aacgaacaaa taatatctcc caaaaagaaa tcaccaaaact
1201 caacagggaa aaaaccagac atcatcagtc taataagagt aaccaccagc cattttctag
1261 tgtgtacaac tttgtcccaa tatggaatta gaccagaggg acataaaaaat aagaaagaca
1321 caataccaat atataaaaaag cctaagggtg tgagcttctc caaagcgaaa cttaacatgg
1381 aacagaccgc acaaggtact atgaagaatg tcaagaaata caacttgagc ggaattttac
1441 tcgtggcaaa atcattgttg aaaaattgcg taccattctt ggattgaatc tcaccagca

```

SUBSTITUTE SHEET (RULE 26)

1501 taataaatct ataattaatc ccggttctat gccagataaa acaattaaca agaaagagaa  
1561 acgcgatcaa caaaacccatg taccaaccac cccacagtgg gaacagaatc ttgtgcgtaa  
1621 atgacgtttc ttcagagcta atgccccaaat acaaagtgtg agtgatcaaa gtcattgaca  
1681 cacctatgcc cagcccgaca accagcatct gcacaatcga tctgtgtgtt ctatgaacca  
1741 tttgttcgga gatagagtac tgaatgggtc atttcttcaa cttatgggtg ttgtgcttcc  
1801 tgtccttagg cgaattgggtc aacgcagtgg tgaaccactc ggtgatctgc gtttccaacc  
1861 aagtgatggg ctctttgtct ctttgggcgg aagagagctc cgaagtgggt gttgggtgcg  
1921 aactggttat ttgttgcatc ttctgtgcc acaactgcac gttggcatcc gcgtgcttga  
1981 aaaggggtga gtgggtcctg gcgtacgaca tgaacgtggg cagctctctt gtgtgacacg  
2041 ttttgtcgaa tttcttgacc attttacgga acccggtgac gttgatattc cggaacgatt  
2101 tgaccaattg caagtacaga tagtactcga ttatagcgtt gcttaacaag tttctagcct  
2161 ggggtgtcgt catagtctcc agaaaagaag cgccaaaagc aaacgtctcg cggcctcttg  
2221 aggacgcac ctgtctcaag tcctgtagca gagaaaatcc acgtttgggc cagatggca  
2281 gcagcctatt gtccttaaga actttctgac agtacgctag cagcgacaaa cgcggttct  
2341 tggcctcttt ggtgcagggc attgacccat acataacaga ccggctatcg ctgtcgatgc  
2401 tattgaccct ggagtcagaa cgaccagcaa gccctgccgc atatagactc gtggacatgt  
2461 caacattcga actagaccgg ttcaagttgt cgcgctcata attcttctgc agcagagtaat  
2521 agtgcagttg cgactgtaaa acctcaaact tcttgtcgca ctctttagc aaccaaagg  
2581 aaaactcgtt acacttactc aactggaacg agatcaacca gtcttcaatg aagtgcagaa  
2641 cgaactccct ctgcaaagca gaatagctct tcttgaacgc aggtccggag cgatagtctc  
2701 cgtegtcgc acttttgccg ggctctctct gctgaaatgc agtctggtat accgacacag  
2761 agggcatcca gctccggtag gagctggatt gctcttcttc ggcattccagc ttctccttgt  
2821 agcggcgaag cttcttcttg ccgaccttat aatcaatata tttgtccctc cattccggga  
2881 tggcagactc ggttagatgg tcagcaaact tcatctgata cggtgctatg gtaaagccct  
2941 tccattgctg ccaactgaat taaaaaaaaa aaaaaaataa ctaccaacca tcaaatcaaa  
3001 caaaacaaaa caaaaaagca aaggagaaaa cccatcttgt acatgtacag tccccactg  
3061 ttgcgaaaacc gtgcgatgat gttcatggaa ctgtcgtaaa ataaaaccgc atcccgtcc  
3121 acgtgacaaa cggcacccaa aatccgttct agaaatgcct cacaccaca ccgatgatgg  
3181 gcttccgact cgatgactac caacacatag ccagccgag cggctggcag catgtgcatg  
3241 ataagataaa gtgatgatga atgatgggtg taacgatttt gaacaaatgg ctgcagcgat  
3301 agcaactaag gggatggaaa gacagatctg ggagagataa ctgcaggggtg tggcacggca  
3361 acacaaggct attgtattgc actaaacggg caagaagcca tgatgtgctg ctgtatccca  
3421 aaaaaaaaaa taatggattg gcgctgttac tatatatatt catatgtcgt gtgtttgtat  
3481 atgtgtggga gtgattgtgc gtgtatatgt gtgttggcgt gtgtggtaca atgtgtgtgt  
3541 tttaatgtag aaatgaggtt gtgacacgtg atcgggaagc cacagtttgc gcggagatat  
3601 tttatttttt ttcatcagcg taagaagaaa gcaaccttgc agtctgtatc gtaagagaag  
3661 actgcagtta aagaagtta gagaagagg catgaggaga gagaggcaaa ggatgatgag tttcgaggac  
3721 tgatttataa aggagaagg acgaccttga caatagtaat agtaataaca gcagtgaat gacagatag  
3781 aaggacaagg acgaccttga gagattgctt attacgggca gtacgatga tttggcaca  
3841 gcgatgatgc caccattaaa gagattgctt attacgggca gtacgatga tttggcaca  
3901 ggatcatcgg gtaagaagaa gatgacgatg gcgacgaggt cgccatcgtc atcaccgat  
3961 tttggcagaa acgacagcg cactagggtg cagccattgc cagaatataa cttaccaag  
4021 ttctgctatc ggcataaccc ggacattcag ttctcaccaa ctcatacagc gtgctacaag  
4081 caggatttga aacgaacgca agagattaat gctaatatcg cgaagctacc cctgcaggag  
4141 caatccgaca tccaccacat tatctcgaag tacagcaatt tgcttccac agctttccta catttcgtca  
4201 ctgattcttg atggatcct atcgacagt tgcttccac agctttccta catttcgtca  
4261 ctggttacac acatgatcaa gatcgacttc atcagcatc tgccgcagga gctgtcgctg  
4321 aagatcttga gttatctgga ttgccaatct ctttgcaacg ccacgagagt gtgccgcaag  
4381 tggcagaagc tcgcggtatga cgacagggtg tggtaccaca tgtgcgagca gcacatagac  
4441 aggaaatgtc ccaactgtgg ctgggggctg cctcttttgc acatgaaacg tgcgaggata  
4501 caacagaata gtacaggatc tagcagcaac gcagatatcc agacgcaaac tacgcgacct  
4561 tggaaagtca tctacagaga acggttcaaa gtggagtcaa actggagaaa gggccactgc  
4621 aggattcagg aattcaagg ccacatggat ggtgtgttaa cgctccagtt taactacag  
4681 cttttgttca caggctcgta cgactccacc ataggtatat gggacttatt cacggggaag  
4741 ctaatacgaa ggctcagcg ccattcggac ggctcaaga cattatatt tgacgataga  
4801 aagctgatta cgggctcgct cgacaagacg atccgtgttt ggaactacat aaccggtgaa  
4861 tgcatttcca cgtatcgagg ccactcggat agcgttctga gcgtagattc ataccagaag  
4921 gttatcgttt ccggcagtg tgacaagacg gtcaaggat ggacagtgga gtccaggaca  
4981 tgctacacct tgagaggcca caggaatgg gttaattgcg tcaaatgca tccgaaaagc  
5041 ttttcatggt ttagtgtcag tgacgatacc acaatccgaa tgtgggatat caggaccaat

SUBSTITUTE SHEET (RULE 26)

```

5101 tcatgcctaa aagtgttcag ggggtcatgta gggcaggtgc aaaagatcat accgcttacc
5161 attaaaggatg tagagaatct agccaccgac aacactttctg atggcagctc tccgcaggat
5221 gacccaacaa tgactgatgg tgcagacgaa tcagacacac cgtcgaacga gcaagaaact
5281 gtcttagatg aaaacatacc ttatccaaca catctactat cttgcggact ggataacaca
5341 atcaaactat gggacgtcaa aaccggtaaa tgcataagaa cacagtttgg gcacgtggaa
5401 ggtgtttggg acatcgccgc tgacaacttc agaattataa gtggttctca cgacggaagc
5461 attaaaggtct gggacttgca aagcggaag tgtatgcaca cgttcaacgg tcgaagacta
5521 caaagagaaa ctcagcacac acaaacacaa tccttgggtg ataaagtcgc ccctatcgct
5581 tgtgtttgta ttggagattc agaatgcttt agtgggtgat aatttgggtg cgtaaaaatg
5641 tacaaattcg atctcaatga ttaggacctg tgtgtggtct tttcttggtc aaaaacatcc
5701 gtagtacctc gaatatatat gcttacatat ataatgaaaa atacataata gcattttaca
5761 tttattttat ttccaggaaa aaaataaaatc gctagcgtat cactagcctt ttccatcttc
5821 agtttttttc ctctctcttt ttctgtctga taaaactctt gaccattaga tgctattact
5881 cccccacgga gcccttccca cgctaagggg gatgtcaagg gggggactga agataaagct
5941 tattatattg acggcgatga tgaagagggc gtacgcataa aatgtcgtac aagatccata
6001 atctgcaata ttgttccctc aacagtacta ataaatccaa agggcgaagg tactttatta
6061 tataaacgcg cacggccatt gtatataaag gcgattacat aatagaatac ttatcgattc
6121 cgtaatcctt gaaatataga atatacataa aatagacaag aaccaacag ttgaggcgcc
6181 agctttggta atagcttaat aataaataaa aaaagataat atagctagct agtattattg
6241 cttttatccg tatggccacc accacgcaac caaaaatat actgatggat gaacctttaa
6301 atcttcctaa taacagtgcc cacaacaata actatggaaa cataaatgcg aatataagaa
6361 cttttgctgg tatgagcatg cacatgcacc ctgccaggct gaactctctg gagtttttgc
6421 acaagcccag gagactatct aatgtaaaaa tgcacagatt acctcaggac gagcttcaaa
6481 gaaatacggg catgaataag ggaatgtatt ttaatgggaa acaagttcat gcccatcacc
6541 cgtttataaa ttctggagcg aactttaacg cacatcatca agacgtcagt aaattaggcg
6601 aggaggaaga cgaaatctct cctctatcac atgataattt ccagtatgaa tccgaggaaa
6661 atggtaatcc ttcacctccc atttacaaga aatctggaga actggttaag agttcattaa
6721 aaagaagatc caagtcctta cccattactc ccaaatctat attcaacaaa actggctcta
6781 agagtaaaca tgtcaattta gatcatgtag atactaggct attgcaaaga agtaaaagtg
6841 tccatttcga tcgtgtttta ccaataaagc tgttcaatga aaatgagaaa cccatagatg
6901 ttggcaaaca aatggttcaa caagatgttc tgaatttcaa gcataagcct ttgacgagac
6961 taagtgcctt taatggcggg agtगतagcg tacctataga agatttacta tctgaaaaca
7021 accaaaacga atatggagat acatggctac aaaaccgaa ggggtgattc ctatttggtg
7081 caaattctaa taatcgtaga aataaaaaaa agaagttaa actaagtgc actatgagtg
7141 atattgaaaa tgacaatgat agtgacgacg ctataaacgg tttagtgaag caacaagaca
7201 aagaccaagc tcatcttgcg cacgggttga agaatttggt aataaacgat gacgacgatt
7261 atttagaaac aagaacaaat tctgtctaat caggagccaa cttgtttatt ggaaactcta
7321 aaagaattgt tggctctttac aacaagaact ttccaatatt aagtgcagg aaccgtaaga
7381 gttaaaaact taacatattt ctgaatcttt cccgtggaag gcccgttttt ttgcaagaaa
7441 ttacgtact aactggcttc cacaacatgg ttataattgg caaagtcttt gtgaaaaaca
7501 tatactttga taagaagatt atcgtaagat atacatggga tgcattggga acttttcatg
7561 aatcagaatg cgtgtatttt tctaattgca atggcatctt accaggaagc aatatggata
7621 tttttaagtt ctccattgat gatatacaca atccaaatga taaagatagc aatatatcac
7681 aattggagtt ttgtattcaa tacttaactt ggggcgttga tcgttctagg aaggaaatatt
7741 gggacaataa tgattcgga aactataaaa ttgacgtggt aacgaatgaa acgaggacag
7801 ggcccaaac agacgtcaat gataactacg agatgaaaca tagtcttttc agaaacccat
7861 tccattaaag aaaaaacaa aaactaatag tattgtttta catatacaaa agaaaaaaaa
7921 aagataagaa tacgtacttt ctatttttat ttgaaggcca ttttctttat ataaaataat
7981 atactggaat gatgttcttt ttgttgcttt ctatatattgc tttctttttt tactttttat
8041 tcaactccag acgttcttaa ataactcggt atcatccaat gaaatgttag aactggaatt
8101 tgaattcata ctattattat ttgtagcggt aattccagtg ttcacgaatg gtgacggagt
8161 attacaaggc tgaggtgtcg tggagtgaa aaatgagttc tggctttgag cctgggcaga
8221 aggttttgag tataaagata aaatggattt tttcaaatcg ttcccttgcc caaattcacc
8281 gactctgttg ccagtcttgg tattagcagc acctatgctt gttgcagaa ctaattacact
8341 tgtattagat gtagtcttag aaagcgatga aacctgtagg tttaataagc tcgtattact
8401 ccttgagggtg cttaataagt gggaagggtg ttgtgtttgc gtcttttgta atgaatttga
8461 gctctgggtc aatcttgcat tcgaagcagg caacgaatga ttcgcagacg gcttatgcag
8521 aactgggtca gtagaatcat ttaacccttc aatactggag aggtcaccaa tccacttctt
8581 atattcatat ttattcttga tgaaattttg taaactgctc gtatcagtga ttttctctg
8641 tttcaattca tcagccaaag tagcttcgta gtatgagtta gctcttaaat tgtttttgaa

```

SUBSTITUTE SHEET (RULE 26)

12/21

8701 ctgtatcagt ttcaccaaatt gttcttcctt ccatgtatcc agatcaacag atttcacttt  
8761 cgaaatgtgc gttcccaatg atctatgtat accagcacat ttaatgcaaa tgaaaacacc  
8821 aagtgaccag gaagcccagc gtggatgtag ttgcccctta cagtcggcac aatgactggt  
8881 tcctggatcg cgtaaaagag cacttaatgc cttcttgact gggactgacg tcgacatcgc  
8941 ttgtaaattg cccacagttc tatgtctcctt tgtacagttg aagcaagtat tacctttact  
9001 tttgttacta ttagtaccga tcttatacgt attatcgagt cgaaaatcgt ccagcacgaa  
9061 atatggaaac ggtgctgtta ggtgactaaa cttaaatacg tatatattat atatgttcca  
9121 atctatatat atatatatgg cactctgtta cagttcctta tcttaaaaca caaacacctg  
9181 gtcttccatc ttggaaagcg gtttggaaac tctgaacccc aagtcacagg tacttcttct  
9241 aactgaggca accatggctg gaggaccaca gatcaaaatt tgaacgttgt ccattgtagc  
9301 agcgggcaag tggtccttga tgacatcctt ggtaatgtat cctacaccac cagtcacagtc  
9361 ttcacgggtc ggagagtcta agtagtaaac tatcttaaat tgggaaggct tcatggccac  
9421 caacgcttcc agttccttct tcaacagaat atcctcctca tggacgttcc caaagactag  
9481 agagacctg gtagtgtcgt gagggtccat ggcaatagct tcatgatct gatacatggg  
9541 cgcaatacca gtaccaccag caatcatccc tagatgggaa cggcagtttc tctcataatg  
9601 atagttccca cgagggccct tgatctggat cgagtcacct atcttcaact ctccaatcat  
9661 cttagaaacg ttacctgtgg ggtaagactt cactagtaat tcaaagtttc ctttgtatc  
9721 tccatccaac gatgtgggcg tatacgaatc ggtaatatcc ttaccattga tattggcctt  
9781 aattacgata tgctgaccaa ttggtaaacc gagtacgtcg tcagcatgag gtacccgaa  
9841 cttgtacatc gaagtattat gcgttaagat gggtttttca accagcgaa atgattggaa  
9901 gtcattcctt ttgggatcca gcacaggctt gggtctcggt ccgataatga acttgaagag  
9961 caaaggcacg accacgatca cgatgaccac cacaagcttt tgagcatcaa tagccatctt  
10021 cgattgctta attgattgag tctctgttg aagagccagt tgaatgcata ctttcaacac  
10081 ttttttttct cgttcatttt ttgccttat ataataagaa tatttataca tatgtagata  
10141 atttaaagaa gtagaaagag cccttaatgg taccgcttta cgaggaaatt tagcactggt  
10201 acttttttga gagtagtgca gtcttagaag ggcttttgag cttgatgtac acgtccgtgc  
10261 cccagccaag taaactttga acgtcaatct tgccccaaa cagttctagg tacgtcttgc  
10321 acattggtag accaaagccc atgccggaga cgttggtgat ttgctcgccg ggcagatctg  
10381 tagactcaga gtcagctgat tgttgggtat gtgtagagta agagtattg aacatgagg  
10441 cctctacctc gggcgtgatc ccaccacat ggtcccgtat ccgtaagtac agctcgtcat  
10501 cgtctggctt taaaagattg atctctatgg gcatatgttc ctttccaaga gctatctgag  
10561 cttcaaaaagc gttcttgaat acctctgtca taatatactc cagaatgggc ggaatgcacg  
10621 tgaatgtgat gtcttgtgat ggcgggtgaa ttaggaccgg ggtccgctga gtgttgaact  
10681 tcacaaagca tatatcattg acataatcag aaacgtgctt gatcaattgt gctatgggga  
10741 ggtcccgtg caagattcca atcatccttt tgttcgtgct acctttgttc tgtgccatca  
10801 acgacaaata gtgcgtcaca agcagcttca tcgtgattct ctcttcagg tggaagtcca  
10861 agaactgcca aatttgaaac tttgggtaac acgattggat ttctgaaga ctttttgcca  
10921 ataccacaat ggcgtcctcg tggctgtcca gcagttctgt gaacttggcc tgtatttgg  
10981 gaggattgtg caactcgtac gggtagcgga ttgatagcaa cgtttgcaag cttttcaaat  
11041 acaaagagtt ggttctctca atatgagggt tgattaccgc attatacggg agcctctgaa  
11101 tggcattaag ccgtttacac gtcaatgaaa gcaacagggt gattgtcttg atcgtcagca  
11161 tgtactcctc cttcttgggtc agcggcgcc tgtattgcag gaaatactcg tagtttaggg  
11221 gcgcaattgg tttactagcg tagtctgtga tcagcagctc aatgttcgac ctaatcttgt  
11281 aatgctggtc gaacgacagt tgcgacagca gctcgtgtga gggctcgtgg cgatgtgccc  
11341 agcgcattcc cccacatttc catgaacgca taatcttcca catactaccg tgaaggcgtc  
11401 ttgataccac gtcttccagt tatggctttg actcaccaat gttctcagtt taatgttgtt  
11461 tgcttcatac tggcagacat tttcccttat taccgtgaga agcagcgggt ggattaatcg  
11521 ggatgtcaaa aacaagaaaa ttgcaacctt ctccacctat aacctatatg attttagggc  
11581 agaggagtgg aataagcaac aaatcatagt catcaatgtc gtttaatgcc ttcgccagct  
11641 ctttgagcaa gaaattacag gaaatatcca caagtgtttc cgaaaaaacc caggaaattgc  
11701 ccagtttggc gcaatccacc caaagaatgg tccagggaacg tttgggccaag gtgacggaca  
11761 tctcccaatt gccaaagagag tacacagagc tggaaagataa ggttgacacc atcaaactga  
11821 tttacaacca cttcttgggt gtaactgcta tctacgaaaa cggatcgtac gattacccta  
11881 aatacatcaa cgaatccgtc aacgagtttt caagaagcgt ggcttccaag ttgacagagt  
11941 tgactcatgc tacatctgct tctgaggcac aaaacatctt agttgctcca gggcccatca  
12001 aagaacccaa gacgttgaac tagccctca gtaaaagtggc tttgactccc agtgaatgtt  
12061 tgaacaagat gttccccacc gaggaaacaac ccttggcttc ggcactcttg caattcagtg  
12121 atgtgcaggc taagattgct caagctagaa ttcaacaaga taccttgatt caaaccaaat  
12181 tcaataaaaa tttgagggaa aggtctctct ttgagatcgg taaggccgat aagtgcgca  
12241 aggatgtcca ttccatgaga ttaagatatg acgtggcaag aactaacttg gcaacaaca

SUBSTITUTE SHEET (RULE 26)

12301	agaagccaga	aaaggaagct	tccttgagag	tccaaatgga	gactttggaa	gaccagtttg
12361	ctcaagtcac	tgaagacgct	actgtgtgct	tgcaggaggt	tatttctcac	gctaacttca
12421	gtgaagattt	gaaggaattg	gccaaaggctc	aagctgagta	ttttgaaacc	tcggctggcc
12481	taatgaaaga	gttcctatcc	aactcatttg	cggaagagcc	ggaagcaaa	cctgaggttg
12541	cagaagaaga	aaagccacag	acagctatct	ctatgaatga	cgaagacgac	gcttaatagc
12601	cgccctgttc	ttgtctttct	ctctccttct	ctatatatat	atatttactt	aatacaatat
12661	catattctct	agtgtcgtac	gaatattatc	atatttttgc	tggcttttgt	agtgaatata
12721	ttttttgtag	agaaaatata	attactggaa	gagaaaaagc	tactgaaaaa	aatttcgtaa
12781	cagtcaccaa	agggcaaagt	gagcaaccca	aaaatggacg	ctactcaacc	gcaatacgaa
12841	ctatccgtag	taacacaatg	cctaaagtct	gccattgacg	tcatccaatg	gttgattcct
12901	actattacta	agttcagcca	atcacaccg	ttagttttcc	aactattgtt	tattttcttc
12961	acattttatg	tctttttacaa	gttggtgatg	aacttcatta	ctctgggtcaa	aagattcctg
13021	tatttgacgt	tggtagtaac	atgtatcgg	atttatatgc	gtgggttcgca	gcagtttttg
13081	actgtggacc	tggtgaattt	ctacaatttc	gtcatgtcaa	atagatacta	cgcatthaaa
13141	atttatactc	tgtttattaa	tgccctggaa	agagaaatca	acactgttta	tcatttggcg
13201	cagatgaaaa	tggaacagtt	gcttaaatag	agcgaaacgc	ctctgcttac	tcggaacatc
13261	ttaaagtaat	tacatatctt	aattttctaaa	tattcatata	gataaagaac	gttttttttg
13321	ctagattcct	cccctagtca	ctatcaaata	ttgagctatt	tttggtgact	tgtgttttcc
13381	aaattattca	ttacaattgt	gacttgattt	ctgaagtfff	aagaaaaatt	gcaactaag
13441	ttattacaag	cgatttatcc	ggtcgcgatt	gaagaactga	aaacaacagc	agcagcattg
13501	taccaagaat	cccaagatgc	tcaggtgtgc	gggtcaagaag	tttgcataat	ttgccacctt
13561	cctcactatc	gtcgtctaata	tttacctcta	tacttatcca	tctttccatc	cagaacagtg
13621	ttcatggaac	tgctctaaca	agaacgcacc	actacagaaa	gatttgactt	ttgtggacaa
13681	agtgaagaat	tacttcagtg	atgttcgaga	gcaatggcat	ggtagccatg	cctctgcagg
13741	taacgatgag	gatattcata	ttttggcctt	cggtgatcct	cagattaaag	gcactctggcc
13801	aaagacgcca	tatgtgtccc	gattagatac	atacggtaat	gactactatc	ttggtcacat
13861	ctatgatatg	atgcagcaga	gactgaagcc	tcaggtgggtg	actggtattg	gtgatctttt
13921	ctccagtcac	tggtatcggtg	attccgaatt	ccataataga	acgaaaaggt	atattagcag
13981	aattttcaca	agagacccaa	cttctattga	aaatatcaaa	caacagaacc	tcgatgagaa
14041	aggtcaatat	aaggcgaatt	ggcctgaatg	gggagaccgt	tttaatgaga	ttttagacaa
14101	tgtcaaggaa	aacgagggctg	ataatcaaga	actttctttt	gggttttggtt	atgaaaatat
14161	ccactcatgg	aatccagact	tggaagactt	tttaatcatc	aacatcacag	gtaatcatga
14221	tgtaggttac	tcaggcgacg	cgacttatca	acatatgaca	aggttccacg	accttttcgg
14281	taaggacaac	tattggattg	aatacgaaac	caacactacc	catccttgga	gaattgtagt
14341	gttgaatgat	cttttactag	aaggtcccgc	tttacaacca	gaatttggtg	aagctacttg
14401	gatctttttt	aaccaactaa	atgaacgtaa	attcaatggg	agtactgttt	tggtgacgca
14461	tgttccattt	tacaagcgtg	aagggttatg	cgttgatggc	ccagacacta	ggatttatcc
14521	agatgcccac	gccccagaat	catataaatc	tgggcttttg	agatcccaaa	atcatttgag
14581	tgaatccgtt	tcaaatcaag	ttttgaatat	gatatttgaa	aatgggaagc	caggtatcat
14641	attaactggg	catgaccatg	agggctgtga	aacagtttat	aataaaaaat	ctacatctac
14701	ttgggaagcg	actaaaaata	tcgaaagtga	cgttttcggt	aaagaaatca	cagttaagtc
14761	tatgatgggt	gaattttaatg	ggaatactgg	tttggttaca	ggacacttca	acacagattc
14821	aatgacttgg	gagtggactt	tcagtttatg	cccattcgct	attcaacatg	tttggtgggt
14881	cgctaaagtg	tcctgtctcg	tcaccatatt	cacttgggtc	tcgctactat	ttgtctaaac
14941	aaaaaagagt	aaagaagata	aaactcctgt	atagagattt	atattaaatg	tatgtatata
15001	tatgcatata	ctgctcttca	aattgcttat	gtttttataaa	cattgacatc	gggtttccac
15061	acatcaagat	aatcattata	ataccttaga	ttgtaaaaatg	tttacttact	actttaaaaat
15121	aactataaat	aaaattaaaa	atcaaattaa	aaatcaaatt	atactgccat	tagcactgat
15181	ttttgttggt	tttggttttaa	agggatgcac	attttttttt	tttatgttat	tatcatcacac
15241	taattttttcc	ctgtttcaat	tgtttcaaga	gctgcttgcc	gtgagaaatt	tcttggtttt
15301	gttggtctaa	agttacatta	ctctgatcat	taacattggt	attgtcaaca	ctcacatcac
15361	ggacctcaga	cgctgtatct	accgggtggc	gcaaaaaatga	ataatccagt	ctgaagttaa
15421	ttttatcaat	tactgtccaa	tcgtccagtt	taaaaatttt	gtaatcacca	acttcgcaaa
15481	tttcattaaa	aaacttgacc	tcaccttgtc	taaaaaacca	cattgtacca	tcctttgaaa
15541	ctttccaatc	cctctcattt	agaatcttgt	atgcaatctc	cctttctaaa	ggagtaaatag
15601	caaaataata	gttataaaat	aacgaaaaca	tttctaaagt	tctgaaattt	tctagaattt
15661	tggcataaat	tgacgatgaa	tcttctttca	gcctttcgga	gcctataata	atatcacgca
15721	acgaacaacg	cataacgtcc	cactgttgag	tagatctgaa	tgcatccaag	ggagatgggtg
15781	gattaactcc	ttgcggtact	tcgaccagcc	tggaaatttc	acacatatca	cgagaccttt
15841	tgtacgttat	tttcgattca	atgtgactat	tatagagttc	agaactcatt	atgaattctt

SUBSTITUTE SHEET (RULE 26)

14/21

15901 gcacaccact tggtagaagt aaagtttcaa aatcacttac aaattctttt ttgatttcat  
15961 ttgtcttagc ttctctttgt tccaagctta aatatttcgg tgtgcttggg ttgtcttctt  
16021 cctctggctc cgtctctgaa tccctatccg attcaaaatc gtcacgaat actccaaaag  
16081 ttggcacttg caatttatct ctttctcttg gtgactcttc ctcttctgc tccataacct  
16141 tcttagttgt gttctcaggg ccattttggg ttaccgtagt tgcaacttca tgttcagact  
16201 ttggatttgt cgccaaggaa actaaaggat gatgtgagga gctcatattt ctatagaagg  
16261 cctgatttgt ttgatgcaca gcagccggcc cagcagccaa aacagcagcg gccgttgctg  
16321 ctgaagatga tgcgcccggta ttgtcaggtt gtaaagacaa agatgaagta gataacttag  
16381 gtgtattcaa cgcactacca atcctgctgt tagcggttga tgatgttga tagcagcag  
16441 cagttgttgg agtctttgtt gaggtattgg atatggttga cgatgcagaa gtgacttttc  
16501 tatcttttcc taccgcttgt gatgcagcaa cggcccattt taattcacca gcaggttttg  
16561 ctggaagagt agcaggtttc aatgtagtgg caccagtaat accgttttga gtttgatgaa  
16621 tatgtgtatg tacatttgtt ggagtttctg gtgtagctga acttggtgat ttttgcaatg  
16681 acggaagcaa attatcagca ctgctcctgg gactctttat agaagtttta acagcctctt  
16741 caggtcttgg tgcattatgg ataggggaa atgaaggact tctttccgtt tcttttgaag  
16801 catcagcaac gggaaatgaca ggtgacggcg tagaagaggg accagcaacg ggtatagctg  
16861 cacctgttgc atttttttggc gcaagtttag cagccttttt ggcttctctt tccaatttcc  
16921 tttgctcctt tttagaaaagt tttgatatgt cctgcagaga ttcattagca ttcgatgtat  
16981 tgttatcttc agcattttgt gaagcaaaat actggggccac ctcatgagca atggcttctg  
17041 tactctgtaa attcaaaccg gaagtaattt tttcatcctc tacgaaatct ggatcctgat  
17101 ttgactccac cctcattggc taacaatctc aaggctaact ccactctgct ttgatgccac ctatacctcg  
17161 cctcattggc taacaatctc aaggctaact ccactctgct ttgatgccac ctatacctcg  
17221 cttgaaaacg cttatattgt tctttttttt catcatttgt cgtagatgac gttttcttct  
17281 ttttattaag aagtaaaagc ttgtcaattt ccactttagg ggaatcgtat tgcttttcca  
17341 gctcatcgat catttgggaa aggtactcag atatatctct cctttccctt tcttgccggg  
17401 ctaaaagtctc tgattttttt aggtcaatat tagagtatgc tttttctttg gagccttct  
17461 ctacagcctt atacttttcc atcgctattt ctacagacct tctgtaatat agaagagaat  
17521 ctttatcttt aatatcggtt gagcttttgc atgattttat ctgttccctt agcctttgca  
17581 gctttttgac ttctcttttt aaatccgact ctgacttgtc cttttgggaa ggattgtttg  
17641 tgcattgttc atgtctttcg taatagctat tgaagatttc taaaccttgc ttaatttttt  
17701 taaagaccct atcgacctcc tgctgtaatt ttctatgagc catgtcgatc gactaaacat  
17761 acaatgaatc ttcaaaaatc taaatttgaa aatgaataaa taaatgtgct gtagataata  
17821 gctttttgatt ttttgtcttg acacaatgag acttctcggt aaagtcaaac caaaaggaga  
17881 tgaacaaaag aaagttcgaa atatcaacaa aatgctttta aaaatgtaga agcaaagtta  
17941 ttaataactt tacaagcgcc ttagtcagtt tattgatctg taggtcagta ggttttagag  
18001 attacgcaca gatgaaagtg aattaagcta ctattacata aaatcaaggt atctttcaca  
18061 agttttttcac tcagcgaaaa ttgcgagacc gctcgaggaa ggtgacgata atacgatact  
18121 tacaacttta atattataaa tataagcatg ttggtggaaca atatagtcag gcaaactgct  
18181 aaaagtcccc acttataaac agtgtgtaaa aagtagcttt gtttttggctc cttatccttt  
18241 atttggcggt ttgacagtga agacaatgcc gctaccgatg cgttgggtat attaatcctt  
18301 cgattaatta taccatattt ttgaatgtcc agtgcaaatg aagaatttgt attcgttaact  
18361 tegtatgtcg gtaggaaata ctgaccagg aactcattaa ttgaatcgct gcctaacaga  
18421 ggctcaacct gcaggtctat cccctgccaa ttttgagaac tctcattaag atatttttca  
18481 aactgagtggt ttgtgtcggt aaccctaaaga tttaggatgt ccgttgattg cactttcaaa  
18541 ttgggggtcag tttgaaggct taaatttgct gtaatgaact tttcattcac ttccatttgg  
18601 aaattttcac acaataatga tacgctgtca ttaatgaaac cctctatcgt agttgttata  
18661 tttccatctg tccaaaacttc ccttttggat agcgaagggt ccgctctttt gtggaatgta  
18721 ggcaaaactgg gtacttcaaa gtttgttaat cgcgtcacta tctgccaatg aattaataac  
18781 atcaaaaaac caaacagcca caggtgagcc ccatttgaga ttatccacca gttacagtaa  
18841 aatagttttg attttttgac ttttttgtca tctgattttt ctggttctct tttaaatatt  
18901 ttttgattga taagctttgt taattggtag ggaattggat aaagtgtact gagattcaga  
18961 gtagtaatta agcttttcaa aactttgttg tatttagtaa aattggtttg tctgtcagg  
19021 ccattaatat gggacataac caggtttact tgctgatttt ccagctgaaa cagaattctt  
19081 tctgattgcca tgagcaaaat cacaaatgca aagtacatga ctgtgaaaat tattgtcatt  
19141 ctcttacatt tacggaaaac gtgatttttt actgcactta gtacactgtt ttctctgcta  
19201 tttgttttca gagaccgttt aattattgaa gtagacgatg taacctcggt actccccca  
19261 gataaaaatg aaatttcatt ctgtaagttt gcagtaaaagt tctttttcaa ctgaagccag  
19321 tcaagtgaat ttcgtagaaa attcaaaact aaggtactag aggtatctaa ttgagtagaa  
19381 aagtcagtga tagtgccatt gaagttctgc aatgatttca atttccttga gtagttccca  
19441 aaaatactgt caaacatgga gtaatttaac agtaggtttg atgccatttg tgaaatggaa

SUBSTITUTE SHEET (RULE 26)

19501	tccttatcaa	tggtcattgc	gtcttctgat	atttcattcc	atgtgtcatt	tatcagctgt
19561	gatttagtga	gtagttcggt	aaaaattgta	ttgtttacct	caagaacttc	cttaatcaat
19621	tcagtttcca	aacttatttg	gtcgttgata	agtttttggt	tattatccag	tatgtcgtct
19681	gtgtatgact	tgaatttttc	ttgcatatcc	gtacctaccg	tagattcaat	actccgtagt
19741	ttgctttcga	aatactgata	aattgtatta	tcaatgaatg	ttttatttaa	agagtaactg
19801	ttggcagaat	atatggcatg	ctgtgttggt	actgctgtca	ttgtggccgt	gttgtgggcc
19861	gttgttggtg	ctgtcgtgtg	agcagttgaa	atagatgcaa	agggtgagac	ggatatcattt
19921	cgtttgacct	gtgcgtgaga	catttttgcc	attgtaatgt	caatgactga	aaaggaaccc
19981	caaatagtta	aaataacaga	agtaaagaat	atcagtaata	gaggatggaa	aatacaacta
20041	aaaaacctct	gtggaagtga	taaagggtta	attatgtgta	cattgttcat	ctttgccgta
20101	ctcttagttt	tatcgaaaag	tgggtgtagat	tttcagctac	ttaagccatg	ttagtgttct
20161	agttttttgc	tttaccatgtt	atataaagggt	ccaagatagc	atatgtaa	gttttattac
20221	tgggctgaaa	cattttttaa	acatttttca	ataatgtgct	gttataaccg	tacaaggacg
20281	gttataattc	tgaattaaaa	tgtcgcaccg	aatgaaaata	ccaatgttct	tccaaaccta
20341	ttggcgaaaa	ttaagataag	ctgtcgcctc	tataatcaag	aataactcta	actattataa
20401	atattattcc	cagaagttga	aattgtgaca	catttgagta	atatcacctt	acacggaaca
20461	tagttgcccc	cttctccgaa	attgctgaca	atgataatcg	gggattcaat	gacaatgaca
20521	atgaaggcaa	aagattttca	tattgtactc	ttgttttttg	agttatcact	ttcaagtacc
20581	ttcttggtta	cagaaataga	accgctatag	aggttatcgt	gtatacgtat	ctgttggtgt
20641	ttctaaatca	aaaactcgct	tttaaagcag	tctttcaatt	ggaagattca	aaaggatcta
20701	aaaagatcta	aagtagtaaa	gaaaaaagta	taagcccaca	cctttttggt	aggataatgt
20761	ttactgggtca	ggagtatcat	tccgtagact	ctaattccaa	caagcaaaaa	gacaacaata
20821	aacgtgggtat	tgatgacaca	tcaaagatct	tgaataataa	gataccgcac	tctgttagtg
20881	atacttctgc	cgccgccacc	accacttcta	ctatgaacaa	ttctgcttta	agtagatcct
20941	tagatcctac	tgacataaac	tatagcacaa	atatggctgg	tgtggttgac	caaatacatg
21001	attatactac	ttccaataga	aattctttta	ccccacaata	ttctattgca	gctggaaacg
21061	tcaattcgca	tgatcgggtt	gttaaaccaca	gcgccaattc	aaactatcag	caggctgcat
21121	accttcgaca	acagcaacag	caggatcagc	gacaacagtc	accctctatg	aaaactgaag
21181	aggaatccca	actctacggt	gatattctga	tgaattctgg	tgtcgtacag	gatatgcac
21241	agaatctggc	cactcataca	aatctgagcc	aactgtcgtc	taccgtaag	tccgctccga
21301	atgattctac	tacagccccg	actaatgcgt	ccaacatcgc	caatacggct	tctgtgaaca
21361	agcagatgta	tttcatgaac	atgaatatga	ataacaaccc	acatgccttg	aacgatccat
21421	ccatcctgga	aacattgtcg	ccatttttct	aaccttttgg	tgttgatgta	gcacatttac
21481	ctatgacgaa	tccaccaatt	ttccaaagtt	ctttgcctgg	atgcgatgag	ccaattagaa
21541	gaagaagaat	atcaatctct	aacggtcaaa	taagccagct	aggcgaagat	attgaaactt
21601	tggaaaacct	gcacaacaca	cagccgcccc	cgatgcccac	ttttcacaa	tataatgggtc
21661	tgagccaaac	taggaatgta	tcaaacaagc	cggctcttca	ccaagcagtg	ccggttagta
21721	gtattccaca	atacaatgca	aaaaaagtta	ttaatccac	gaaggactcc	gcattgggtg
21781	atcagagcgt	tatttactcg	aaaagtcagc	agcgaaattt	tgtaaacgcg	ccatcaaaga
21841	atactccagc	ggagagtata	agtgtatttg	aaggcatgac	gacgtttgcg	ccaactactg
21901	gaggtgaaaa	taggggcaaa	tctgcactta	gggaatctca	ctctaactct	agcttcaact
21961	caaaatctca	aggatctcat	ttaaatttag	cggcgaacac	acagggaaat	ccaatccctg
22021	gtactacggc	atggaagaga	gcaagattgt	tagaaagaaa	tcgaattgca	gcttcgaaat
22081	gtagacaaa	gaaaaagggt	gcgcagctgc	agctccaaaa	ggaatttaac	gaaattaaag
22141	acgagaatag	aattttactg	aaaaagttta	attactatga	aaaactaatc	tctaaattca
22201	agaaattctc	caaaattcat	ttacgtgaac	atgaaaaact	aaataaagac	tcagataata
22261	atgttaatgg	cactaatagt	agcaacaaaa	atgaaagcat	gactgtggat	tcattaaaga
22321	tcattgaaga	acttttaatg	atcgattcag	acgttacaga	agtggataaa	gatactggta
22381	agatcatagc	catcaagcac	gagccatact	ctcaacgttt	cgggaagcga	actgacgatg
22441	acgatataga	tctcaagccc	gtagaagggtg	gtaaggatcc	agacaaccaa	tcattaccca
22501	attctgaaaa	gataaaataa	caaagttttg	gtgagcacia	cgggtgtttca	ttcaagaatg
22561	ataaggacaa	ctatatatgt	aataacacgtg	tataaatact	cgagctgcac	cctttctttt
22621	ctgccgttat	tatttcgttt	agttactaca	tttatgggtat	aaattacctt	taagacatta
22681	ttattttgca	tgtatgaaat	ttgctcgtgt	caaccctta	gtcctccttg	cctctgttgt
22741	atggaggaag	cccttttaac	ctgtatttag	tttttttaaa	ctattagaat	taagtacaat
22801	tgtacagatg	gtaaaaaaaa	gtaatcggtta	tatcgtttgt	cagtgttttt	ttttttttta
22861	tttcattcat	tattttattc	ttatttttca	atttggtccc	ttattggggc	aaaccacggg
22921	tgtcccatcg	cttccttagc	agttaatctt	tcttgatgat	catatctcaa	aagattgtca
22981	ataagggtcaa	taatttcatc	gttgccgctt	aaatgtttat	taccatcatt	gatgaatcta
23041	tgccaaggct	ttctgatgta	ttgggtccata	tcgtaaaatt	ctctcggtaa	ggtaattttca

SUBSTITUTE SHEET (RULE 26)

23101 tacttcaaca ggtattttctc aaaatcgctt gtaccaagta ctttgacgat cttgacaagc  
23161 tggctctgtg tactcgttcc atggaaaaat ggctctcttt taaagatcat agaagccaac  
23221 attgtcccaa acgaccacaa gtctaaagaa taatcatata ttctgtagtc aactagtagt  
23281 tcaggacctt taaaaaacct cgacgcaaca cgaacattgt attccatatt aacatgataa  
23341 aattcagcaa gcccccaatc tatcaatcgc aattttttat tcttatgata aatcattacg  
23401 ttatgagggt taacatctct atgcattatt cccattgaat gacaatagtc taaggccttt  
23461 aataactcaa acatataaaa cctaatttcg agatcgggta atttagggtta aagaatacgg  
23521 aagtcacat tatctacgta ttccaagacc aaagccggag tttttgagat gggatccttt  
23581 attatatcaa atagatgaat tatgtttgca tgaccattgt gtggctgata aaagatatag  
23641 ggcctgatga attttaaaac gtctctcttt tgatttggtg aatattgata tttttgaaat  
23701 ggcaaagtcg ttggaggcac tttttcggtta gacaaatccg ttaagatttt gatttctctc  
23761 ttgatcttct tctttttaac tgggttcaac atcttaataa caattttaac ttttagagtct  
23821 aatttgacac cttggaaaac ctccgagtat tttctctgcc caactttatt ttcaatttca  
23881 tagtcctttg tatttggtga ccaatcaatt acagtatttt cataatccca atattcctcg  
23941 gttctttgtt tattgatatt cgtataaaca cgagcctctg accataccct gcatttcata  
24001 gttcgaatca aatattcctt ctacaacccc ctatttttgt ctgtatatatt gacttcttaa  
24061 gtccctattc agaaaactcc tagtaggtta ttgcaatata ccgagtatca acagtatatg  
24121 aagtccttta atccgtttta ctacaggact gcgagggtcg tctaattgct actgaaattt  
24181 tccaagcgtt ggtcatattt ttgatttttc gctttttctt cagtgttcgt tccgagtgat  
24241 ttttgatagc gcttttctac ccttagtgct tgctgtatgt atttatatga tataccagcg  
24301 atacatgtaa atatttctat aaagactgta aacctctgat tacctctgcc tgtgcgtctt  
24361 tattcgcttc ctccagcagag gaagcaatgg ctgcgttctt tgtttggtgg aagatatctc  
24421 ttgtcttttc gaaataaaca gtttccaata gatttctcat ctgagactca atgtcctcaa  
24481 ttaaggaacc caaattagcg acatgtgatg tgaaaataac atctaattgga cgggacatat  
24541 caatagcgat gtcccttttcg gtctgtcttg tcagggtccc ggataacatc atatgagaat  
24601 tctggtcagt ttttggtttg tccaagtgca gaatgattgt agtggtgact ctatagttga  
24661 aactgtcggg agaagaagga gatgttgtaa cttcaaaaac atggatactg tcccaattac  
24721 tgtgatctga ttgggttttt ttgaaaagca ctaccctctg ccttcataa tagagatctc  
24781 cttcttccatt gaggtcccag aggtacacgc ttggagatacc gccttcataa tagagatctc  
24841 tgtaaacgtc gaaagagtca ttggctaaga tctccaattt tcttaaaggg gctgagggaa  
24901 aggggctgtc ttgtagatcc tttggggata gttctgggta ataagtgttc gaccaaggcg  
24961 atctgaacga atcaatatca cgattatagt cgcagcataa gtactcccgg tttgaatcgg  
25021 cggaatcttt ctgtgtggat aggggaacgt ctactgaaga tagtaaatct tgtgccaaat  
25081 ttgggtgtaa ttcgatcaag ttgttttagat tctcctgtaa cgtggtagga tttagcctcc  
25141 taagaagatc taaagcagca tcgaattgag catcagacat tacgggtggtg attgtagagt  
25201 tggagagagt ttttgatgt cacttgattg aacagttctc ctttatttgt tacaattgct  
25261 ctcatTTTTA cgttttccgt ggtgaaaaaa atgaacatat atacatgtag  
25321 taacagcagt agtagatgaa gaatttagag tttaaacagg taacataacg ctataagggg  
25381 aagggaagtg gagatgttat acattatatt tacgtagacg ttgaagggaag aagaaagaaa  
25441 aagggaacag aagaagaaga aaaagagaaa ggaaattcat gtggatttaa gatcgcttcc  
25501 cctttttact taatgtcttg taggatcatt gagctttaat acgtctactg caggacccag  
25561 taaacgttga aaaccacttt tccccaatgt ggcaactttg gttctctttg tagcagtcac  
25621 agtggcctgt ctgggcaaat cgttttagcaa ggccacttca ccgaaataat catggtcttt  
25681 cagtttattt atgacacctt ggcccttctt agagacgtcc acagctccgt actcaattaa  
25741 ataaaagttc tccccttgat caccctcgcg aatgattgtt tcaccgggct ggtagatctt  
25801 ggtatccagt gcatcggcaa gtttggcacg gtcgtacgta gtcaaaactct tcaaaaactgg  
25861 catgctcttc aaaagatcgt catacatgag tctcttcttg aaagagctgc ccaaaagtat  
25921 ttttctgaag gtgagcctgt ctagagccca caacaaacag tcggagggtg ctacaacggg  
25981 ggcagcacga gggctgttgt acataagagc aagttccccg aaactggagc ctggcccgga  
26041 agagttgacc ttgttgctgt tgacgtagaa gtcaacagta cccttttcga cgacatagaa  
26101 gtagtccctt tggtcacctt gcttgattat cgtagcacct ttggggacgg acttctcttc  
26161 cagacaattt atgaccagcc tttttgagtc ggaatccagc ttgttgaaac gaaagtattt  
26221 acggatcgat ttttccagtc tttgcaattg ctgctcggac ttttcttat agtgatcgg  
26281 agtccaatcg tcaaaattgt ttgggtgtaa ggtctcacca tgatgtcttt tctctagtat  
26341 ggcgttgaa gtcattggga gtggaggagt tgatgtcttt tctctagtat gctgtgtgtc  
26401 ttcctgtgct tgctgggtgag tgcctgttc gtgcgggtct aaattaaacc cacttttaaa  
26461 cacgttgag tgtgggtcct cgttcacaaa gggggatttg aacataacac tcgatctgga  
26521 tcttgattga gattgagctg attgaggtct ggaaaatgac tcctctgggt ccggaaatag  
26581 aacaatgttc tttgccttaa attcaggctc cctggccttg aggaacgctc tctgtgtgtc  
26641 cagcctttta ttgaaatagt tggcggagaa ctgaagaaag tcggacggat tagcggcgtt



```

26701 gatttcggttc tggaacagtt gcaattcggc ttgcgattcc ttgggcaaag aagataccat
26761 cgttttattct tactgttgtc ttttgaaaat aatctgcttg ttgtaaatga tcttcctatt
26821 tatgtatgcg tgaaatgcgt gtaatggatg tgatgatagc gatcgcttga cttagtcgag
26881 gaaagcgtaa agtgccccct tttctctttc ctttttccct gtttgttcaa attttctctt
26941 ttctaccttc ctcttcggtt tgctccttgc cagcaaaacg aggaaggaca agaatttttg
27001 agagcggcgg cgcggcgcca taataaatgc acttgagcaa atgcaataag ttcagcttat
27061 ggcttctgca acaaaagaaga ggtcggaatc cgagcacagt ataatacaata tattatcagg
27121 cagaacaaaa gtggcgggta taacacgaag aaagaaggaa ggggtggtgat tgggtgcttt
27181 gtttttttcc gctcgtcgtg gtggcatgcg cttttttttt cttgggtgaa gattagccgc
27241 cgaagtcgta tgctcctctt ttgagcgttt tgaaagatag aaaaagctg agtaagtaaa
27301 atcgtcggcg gctaactctga aaagtatata aagtccttta tttttccac tcgtattctt
27361 tccccctctc cttttttttt ttactttctc acgtatggct tgtttcattt attatcagat
27421 cagtaatcac gtgccgtgtc gttagaaatg acatttggtt ctttgccctt tttgtccttt
27481 ttctatcttc ctactaagag aaactattat acaatacaat aagagaacat cgtgaaacaa
27541 cataaaagat atcacgtcat cagagagaga taaaacatca caaccaatta cattacagt
27601 tttcggttct ttcttagaga gagaaataaa gagcgggata aaaggacact acacagtata
27661 ccccataccc aggtgcaata atacacgtat atctatgttt attgcacatc aaacccaca
27721 tatatgtctg ccagaaaacg caagtttaat agtctcaaac cgctagacac cttgaacagc
27781 tctcgtgcca gctctccaag gtcctctgct tctctaccgc ccaaaagata taacactttt
27841 cgtaaggatc cgaaaatcgt tgatcatcta aacaatgctt ctacaaagga tttcttacca
27901 gtttttgagta tgaacagcga gagtaagagg cagatcgaat tgcgggataa tgatggtgat
27961 aataatgatg aaggtgaagg cgtcaacagt ggctgttcag atcaagattt tgaacctttg
28021 caaagctctc ctttgaaaag acactcatca ctcaaaagca cttccaatgg tcttttggtc
28081 caaatgtcta ataactctgg gaatggttca ccggaaccgg cagtagcgag cacttctcca
28141 aatggctcaa ttatttccac taaactaaat ttgaaacggc aattttcttg cgttgattcg
28201 aaaacattgc gaatttatcg gcataaagca ccatgcataa tgacttttgt tccagatcat
28261 aatcatccga aattttcatt gtattttcaa caatcgggtg tctacaattc acaagttaat
28321 ctgcttgatg atgttgaatt gataatttta gataagaaga actcttttat ggctataatt
28381 ttaaaagatc tgaaaaaggt caagatgata ctagacgtga ataactcttc aatcaacatt
28441 aacacgaaca tcttgatatg gtccactgca agctccgctt caaataaaaa aataaagtct
28501 attaaaagat tctgttgat gtcataattct tcgtcgataa aagtcgaaat tttagatcat
28561 aaagagcaga ttttgaaaag actaaaacat ctgattcatc ctatttcttc gtcatacctt
28621 tcattgaaca tggaaagggc aataaactcc actaaaaatg cattcgactc ttaagactt
28681 aaaaaaacta aactttctac taatgatgat gaaagtcgcg aaattcatat tcatcttcta
28741 tcaaacaaac ctcatgggtt gcaatcctta acaaaaagga ctgatttgct cagccttggg
28801 aaaaaagagc attcaatatc tgttccaaaa tcgaatatat caccctcaga tttctacaac
28861 actaacggga cagaaacttt acaatcccac gcagtttcac aactaagacg ttcaaataga
28921 tttaaagatg tttcggatcc agcaaaactc aattcaaatt cagaatttga tgatgcaact
28981 acggaatttg aaacaccaga actgtttaaa cctagcctct gtacaaaatt caacgatggg
29041 tcaagctata ctataacaaa tcaagatttc aagtgtcttt tcaataagga ttgggttaat
29101 gatagcattt tggatttttt tacaaaaatt tacattgaat catctattga aaagtcaatt
29161 atcaaaaagag agcaagttca cttaatgtcc tctttttttt acacgaaact aattagtaat
29221 ccagcagatt attattctaa tgtaaaaaag tgggttaaca atactgattt gttttctaaa
29281 aagtacgttg ttataccaat taacataaag tatcattggg ttagtgtgat tataacaaac
29341 ttggatgcga tcttgatttt tcatcaaaac aaagataaaa acgatgccat caactccgat
29401 gagatttcta taaataatcc tctgggttaat attttgactt tcgactcgtt gaggcaaaact
29461 cattcaagag aaattgatcc aataaaaaga tttctcatat cctatgcact tgataaatat
29521 tcaattcaac tggataaaac acaaatcaaa atgaaaacgt gtccagttcc acaacaacct
29581 aatatgagcg attgtggtgt tcatgttatt ttgaatatta gaaaattttt tgaaaatccg
29641 gtggaaacaa ttgatgtatg gaagaattct aagattaaaa gtaagcactt caccgcaaaa
29701 atgattaata aatatttttg taaaaatgaa agaaatagtg cgagaaagaa ttaaggcat
29761 actctaaaac tcttacaact caattacatg agctatctga aaaaagaaaa tttatatgaa
29821 gaagttatgc aaatggagga gaaaaaaagc accaatatca acaataatga aatttacgac
29881 gatgacgatg aagaaattca aatcattgaa aatatagatc aaagcagtaa agataataac
29941 gcgcagttaa cctcggaacc tccctgctca cgatcatcca gtatttcaac aacagaaaga
30001 gagccgacag agttgcataa ttccgtagta cgacagccca ccggtgaaat aataactgat
30061 aatgaagatc ccgttcgcgc tgcttctcca gaaacagcat ccgtttctcc tcccatacgt
30121 cacaatattt taaagagttc atctcctttc atatcagaaa gtgcgaatga aactgaacaa
30181 gaagaattta cgtcaccata ctttggaagg ccgtctttta agacgagggc taagcagttt
30241 gaaggcgttt cgtcaccaat aaaaaacgat caagccctgt catccacca tgatattatg

```

SUBSTITUTE SHEET (RULE 26)

18/21

30301 atgccgtcgc ctaaaccctaa aaggattttac ccaagcaaaa aaatcccaca actttcctcg  
30361 catgttcaat ctctatcgac tgattctatg gaacgccaat ctagtcccaa taataccaac  
30421 attgtaattt cagatacaga acaagattcg aggttaggag tgaattctga aagtaaaaat  
30481 actagtggtta ttgttaacag ggatgactcg gacgtcaatt taattggtag ttcactacct  
30541 aatgtagcgg aaaaaaatca tgacaacact caggaaagta atggtaataa tgacagtctt  
30601 ggtaaaatac ttcaaaatgt tgacaaagaa ctgaacgaaa agttgggtga tattgatgat  
30661 gtggcattta gtagtccaac taggggtatt ccaagaacca gcgcgacaag caagggatca  
30721 aatgcacaac ttctctctaa ttatggagat gaaaataacc agtctcaaga ttctgtttgg  
30781 gatgagggca gagataatcc tatactcttg gaagatgaag acccttgaaa gcactcgcac  
30841 gcatagtttt atattttttt ttcttttctt ctttattttt atttttttaa cattctcgca  
30901 taataatagt aacagcaata aaataactag taatatacat actcacataa ataggcatat  
30961 tttttatttt gttctttcgt attcagaatg gtaaattttt ctaatgaaat gttttacaaa  
31021 aatttttttt ctataattct aacaataaga aataaaacag tggaaaaaca tataacttaa  
31081 tgtagatata tatatgtaaa tatgctagca ttcattttta atgtaaggaa gaaaacgcct  
31141 ttaactttca tctggtaaat tttctaaagc tctaccctta gtgtaaacct cgtcttttac  
31201 attctgtaca gtacttttga accattgaat gaacagcaca gttgcttgga aaaagaacgt  
31261 aaatgactct aatgctatca gtagtccgaa ataaatatct tgcaccttg tccatggtgg  
31321 gaatgattcg ttaagcgata aagtgaact cctggatcca tggctaata cgataatctg  
31381 cttttagtaa acatcacgta aaaatactaa ggataaaata ataactcggca agacgtaaat  
31441 taccatgtgc aggaaacctt gtttaaacag ttccaaagcc attgttttca agaagttcaa  
31501 tttactagta tttctgtttc tgaaaaatag tttcaatgac ttatatccgg ttcgaaatt  
31561 gaccattgac gaaatgatcc aaacgacgaa taacaaactc tcgtcatatt taaaccattt  
31621 ggaattacca atagtgtatg aagaattgcc aaacactctg atggtgagat aattgtaaca  
31681 ggctagtata ccacgggta agacgacca cgaaaccata gttctaataa ccgcaatcat  
31741 gatagctata aacaccatca gggtatactt caccaacata ctatcaataa tactgttaaa  
31801 gatggccatg aaaattcctt gacctcttcc cacttctctt acttcgttt cgtcaacggc  
31861 gttgatatct ctctctggta tatcgtgttc cacttctctt acttcgttt cgtcaacggc  
31921 atgttcatcg agagtgtttt ggccttctgt aaaatgaaga atactcttag caatcttggg  
31981 taaacaaaca gatccgacac agacataata atgtttcaaa taggagtagc taatgtcaga  
32041 gagttccttc catgcaacat agaccttatt tctttcacct aacaatagct ttacgactgg  
32101 aagaaagccg aaactacaaa caaagtttat taaagcttga ctaataaatg taacaccaag  
32161 cattaaaatg gaagcaaata accaaaccag gccaaagcaat gtcatatagc gtaaaccggaa  
32221 atctggaggg acgtaaacga tataatattg atcatattcc gtgttttgtt cgtctaaata  
32281 accaaattca ccagcagcac gtttgtttct ctctttaatt ctttccaaat ctaatggttt  
32341 caacagctta tcatcctttg taacggggac aaacattggt tgtacataat ttctggatac  
32401 tatatcagaa gaaggaacac gcattaaaac gccatcgggg acaaaataag catgaacgtc  
32461 acgaacttgc ccaaacaatt ctctgtcttg tctagagtc tttggtttag tgaaaagttc  
32521 ttggtttgac cattctgcat tcttggcagc aatgtacttg taaaacaaat tgcggtaaac  
32581 aatatggcct ctctctgttg gggatctttt acctaagata aaggatgata aacgtaattt  
32641 tcttgaacat aacttaaaaa ttgttttcca atacctttcc aataaggggt taacgtaaga  
32701 gctagactcc aggatcctct tcgtaaaata taaagttagc aaaattgtat tgaacttcca  
32761 tgaaataatt gatgttggtt tataagcttc aggaacggat aataagttcg atttgagcat  
32821 gaatgggaaa aaaattctag tatggaagcc aaaacctaag acaataaaaa tcgcataaat  
32881 aaacatggat aagcaaagcc ttgataattg aatactcatc ggatgaatta aactatcatg  
32941 caagatctta atattaggat ctctctggaga tctgataaag aatagaacac ctggctctaat  
33001 gatatttttt ctaatcattc cgatatattt ggcaaaccaa tacatgtaga gagtaccaat  
33061 cgtccagtat acgaaaaggg aaaatggagg ccatattgag catattgaag gaacccaaag  
33121 catccgacta ttggatgcca ggattgggca aaataatgag aaatctagca ttacaccagc  
33181 tagaatgggg aatccagcca attcgatgaa aaataaagtg aacaccttga aagtgcattt  
33241 gagtgcaaat aaaatctgga aaatcagcct tctagttagg ttgctcattc cgttctcacg  
33301 gccataaccc ctcgagacaa gattggatga agcacaacag atactgactg aggttagata  
33361 cgtcgtttaa gcaggtaaag ctctgatgaa aattgagtgc ttcattggtt tttcagtgtg  
33421 gccgttgtaa agataaatga tgtcatgaat taagtgttct gaaatccaac tcatcgcgac  
33481 atctacttta gggaccagtt tcgtcagccc attgtaagct atatgagcgc cagacaagta  
33541 gtaaagatgg cacaaccac gcaaaataac cttaaatata ccaaaataaa ttttcaacaa  
33601 accgaaccca ataaaagttg gaaacaaata ggaaattgcc aggtatattg cgggtgaaaac  
33661 tacggcaatt atgaaataag caatgacatt caacaatttc aatttgagat ttatcactag  
33721 agggccttgt ccttgatcgt ctgggttagc ctgtgctggt ggcacaccaa cagctgcgcc  
33781 aaagtcttgt tcgtcttgat cgacatttcc agctctgttt tgggcaggtg gtgggatgaa  
33841 aaccggagcg ttgggcctgt ttattgcgtt ctgttgagca gcgattaggt catcaaatcc

SUBSTITUTE SHEET (RULE 26)

19/21

33901 attttgcgca cgacgatttg cccacatgtc aatcggattg ggctctatat gatc

SEQ ID No. 8

```

1 mrrerqrmms fedkdkddld nsnsnnssem tdtammpplk rllitgssdd laqgssgkkk
61 mtmatrspss spdlatndsg trvqplpeyn ftkfcyrhnp diqfspthta cykqdlkrtq
121 einaniaklp lqeqsdihih iskysnsndk irklildgil stscfpqlsy isslvthmik
181 idfisiilpge lslkilsyld cqslcnatr crkwqkladd drvwyhmceq hidrkcpncg
241 wglplllhmk ariqqnstgs ssnadiqtqt trpwkvviyre rfkvesnwrk ghcriqefkg
301 hmdgvltlqf nyrllftgsy dstigiwdlf tgklirrlsg hsdgvktlyf ddrklitgsl
361 dktirvwnyi tgecistyrh hsdsvlsvds yqkvivsgsa dktvkvwhve srtcytlrgh
421 tewvncvklh pksfscfscs ddtfirmwidi rtnsclkvfr ghvgqvqkii pltikdvenl
481 atdntsdgss pqddptmtgd adesdtpsne qetvldenip ypthllscgl dntiklwdvk
541 tgkcirtqfg hvegvdiaa dnfriisgsh dgsikvwdlq sgkcmhtfng rrlqretqht
601 qtqslgdkva piacvcigds ecfsgdefgc vkmykfdlnd

```

SEQ ID No.9

```

1 atgtcactct ctaggtgtcc aactgacaat tcgtcctccc gtataaattc ctctgttctt
61 ttaataaata gcagcagccc tgctacacct ccagaatcat ttgatcctca agtatttctt
121 tcttcactta tcatgggga taacctgctt cctcaagatg atcaaattgc atcggatcct
181 cgctcagaat caaatagttg taatggcaat acgagttctt ccctgccgtg cactgattcg
241 tatcagtacc cattaaagca ttcttgtagc ccttcttttc ttcgaaagt taatgaaagt
301 atagagaatg tctcttataa atgcttagac cactcacccg cagatagtgt tcttgccgat
361 ttttccattt cccttggttc tcaaaggaat tttctatatt ctcatcttc tcttccacct
421 aaaattatat caattgatag aaacaatcga attaaagttag ataatagcat ttcattctaac
481 tccgacaatt tccctccttc tccgaaagtc gacacatcaa acactgtttc acctggtagt
541 aaacctatct ctgaggatct tgaagattta aacttacagt caattgttca aacttttgag
601 gatcttccag aaggaattca atcttatgag ttttttcaac tactccgttc gtgcaatcgg
661 caatcgatgc gtttattatt gaatgaatgc gagccgcttc taaaaaaga tatactttca
721 aatcttccct tttccattgt tcagttctata ttattaaatc tggatataca ttcttttctt
781 tcttgccgctc ttgtttcgcc tacttggaat agaatacttg atgtgcatac ttcatactgg
841 aaacacatgt ttagtttatt tggctttcaa atcaatgaaa atgactggaa atatgttaat
901 ccaaacttaa atcgctccacc ttttttgcac aacgaccaa tctcagatga ctattttccg
961 gaaattttca aaagacattt tctcaataga aaacgatggt tatttccctc gatacctcca
1021 agtcatctat cttttcccat tcatgttcca aactttatga taacttctt actacttcat
1081 aaagacagaa taatcaccac ttcgggatct ggaacaattc aaattcataa tgctattacc
1141 ggtgttttag aagctcgatt agaggggtcat aaagaagggtg tttgggctgt caaaatacat
1201 gagaatacac ttgtatctgg ttcgatcgat aaaactgttc gcgtttggaa catagagaaa
1261 gctaaatgta cgcacatatt taggggacat atttccatca tcagatgctt agagatctta
1321 gttccgagtc gtcttattcg ccatggaggt gaaattgttg aaccagatca accgtacatt
1381 gtcagcggct ctcgggatca tacacttcgg gtttggaagc ttccaaaaaa cacggatcct
1441 ccttatcttc cagataatac aaactctatt gaccgttggg agaagaacc gtattttgta
1501 catactttga taggacatac agactctgta cgaactatat ccggctatgg tgatatactt
1561 gtgagtggga gttatgattc ttcaattcgc atttgagag tttcaacagg ggaatgtctt
1621 taccatctgc ggggtcatag tcttcgtata tatagtgtt tatatgaacc agaaaggaat
1681 atttgcataa gcggtagcat ggataagtc attagggtt gggatttatc gacagggact
1741 tgtaaatatg tgcttgaagg ccatgatgcc tttgttacgc ttcttaattg attccagaat
1801 aggttgatat caggttctgc tgactccaca attagaatat gggatttgaa tactgggaaa
1861 ccattaatgg ttttgccgct taattcaggc tacattagta gctttgtgtc agatgaacac
1921 aaaattatta gtggtaatga tggttctgta aagttatggg atgttaggac tggaaagctg
1981 ttacgttttc tattaacaga cctcacaaaa atatggcatg tcgattttga tgctatgcgt
2041 tgtgtggctg cagtgcagcg tgatgatcaa gcataatttg aagttattaa ttttccgga
2101 tcaagaccgt ag

```

SEQ ID No.10

```

1 mslsrcptdn sssrinssvp linssspatp pesfdpqvfp sslihgdnl1
pqddqiasdp
61 rsesnscngn tssslpctds yqyplkhsct psflrkfnes ienvsykcld hspdpdsvpgd
121 fsislvpqrn flyshsslpp kiisidrnrr ikldnsissn sdnfppspkv dtsntvspgs

```

SUBSTITUTE SHEET (RULE 26)

20/21

181 kpisedledl nlqsiqvtf dlpegiqsy ffqllrscnr qsmrlllnec epllkkdils  
 241 nlpfsivqsi llndihsfl scrivsptwn rildvhtsyw khmfslfqf inendwkyan  
 301 pnlrppflh ndqisddyfp eifkrhflnr krwlfpispp shlsfpihvp nfmitslllh  
 361 kdriittsgs gtiqihnait gvlearlegh kegvwavkih entlvsgsid ktvrvwniek  
 421 akcthifrg h isiircleil vpsrlirhgv eivepdqpyi vsgsrhdhtr vwklpkntdp  
 481 pylpdntnsi drweknpyfv htlightdsv rtisgygdil vsgsydssir iwrvtgecl  
 541 yhlrghslri ysvlyepern icisgsmaks irvwdlstgt ckyvlegghda fvtllnvfqh  
 601 rligsadst iriwdlntgk plmvlpnsng yissfvsdeh kiisgndgsv klwdvrtgkl  
 661 lrlltdltdk iwhvdfdamr cvaavqrddq aylevinfsq srp

SEQ ID No. 11

1 gaattcggca cgaggcggag ctgcgttggc tgcggcctgg cagcaaaggg gcggccccgg  
 61 cggagagcag acccagtagt cggggcgatt atggaccgg cagaggcgg gctgcaggag  
 121 aaagcgctta agtttatgaa ttctctcag agagaagact gtaataatgg cgaacccct  
 181 aggaagataa taccagagaa gaattcactt agacagactt acaacagctg tgccaggctt  
 241 tgcataaacc aagagacagt atgtctaaca agcactgcta tgaagactga aaattgtgtg  
 301 gccaaagcca aacttgccaa tggcacttcc agcatgattg tgcccaagca gcggaaactc  
 361 tcagcaagct atgagaagga aaaggagctg tgtgtcaagt attttgagca gtggctcagag  
 421 tctgatcaag tggaaattgt agaacacctt atatcccaa tgtgtcacta ccagcatggg  
 481 cacatcaact cctacctaaa acctatgctg cagagggatt tcataactgc actgccagca  
 541 cgggggtctg accacatcgc tgagaacatt ctgtcatact tggacgcaa gtcactgtgt  
 601 gctgctgagc tcgtgtgcaa ggaatggtag cgcgtgacgt cggacggcat gctgtggaaa  
 661 aagctcatcg agaggatgg caggacggac tctctgtggc gaggcctggc agagcgaga  
 721 ggctggggac agtacttatt caaaaacaaa cctcctgatg agaacgctcc tcccaactcc  
 781 ttttatagag cgctttatcc taaaatcata caagacattg agacaataga gtccaattgg  
 841 agatgtgggc gacatagttt acagagaatc cactgccgga gtgaaacaag taaaggggtt  
 901 tactgtttac agtacgacga ccagaagata gtcagcggcc ttcgagacaa caccatcaag  
 961 atctgggata aaagcacact ggaatgcaag cggattctca cgggccacac gggctccgtc  
 1021 ctgtgtctgc agtacgatga gaggggtgatc atcacaggct cctcagactc caccgtcaga  
 1081 gtgtgggatg taaatgcagg tgagatgcta aacacattga ttcaccactg tgaagccgtt  
 1141 ctgcacctgc gcttcaataa tggcatgatg gtgacctgtt ccaaagaccg ttccatcgct  
 1201 gtgtgggata tggcttcccc aactgacatc accctcagga ggggtgctgg gggacaccga  
 1261 gctgcggtca atgttgtaga ctttgatgac aagtacatcg tttctgcctc tggagataga  
 1321 accataaagg tgtggaacac aagtacctgt gaattcgtaa ggaccctaaa tgggcacaag  
 1381 cgtggcatcg cctgtttgca gtacagagac aggtctgggtg tgagcggctc ctctgacaac  
 1441 accatcaggc tgtgggacat agagtgtgga gcatgcctgc gagtgttgga gggccatgag  
 1501 gagttggtac gctgcattcg atttgataac aaaaggatag tgagcgggag ctatgatggg  
 1561 aaaattaaag tgtgggatct tatggctgct ttggaccgac gtgctccagc agggactctc  
 1621 tgtctgcgga cacttgtgga gcattctgga agagttttcc gctccaggt tgaatgattc  
 1681 cagattgtca gtagttcaca tgatgacaca attctcatct gggacttctt gaatgatcca  
 1741 gctgctcacg ctgaaccgac ccgctccctt tctcggacat acacctacat ctccagataa  
 1801 ataaccacaac actggcctca taattgcccc ggattcggtta atgttgacgt atttaacaga  
 1861 cctgccaaga ccaggatgaa caacaatcaa actcctaccc ggattcccgg accgatgagc  
 1921 gaggagcagg gctttgagac tctgttgagg acacagtcgg tcagcagccg accaggacgg  
 1981 cctgctcggc accggctgcc tcagtgtgct tatcagaaga tgtctttatc ttgtgtgaat  
 2041 gattggaact tccaagcctc cctcccttcc cctcccttcc cctccctgca cctgtttccc  
 2101 tccattggg ttccagacaa agatgactta taaatatatt tagtgttttg ctaaaaaaaa  
 2161 aaaaaaaaaa aaaaa

SEQ ID No. 12

1 mdpaevlqe kalkfmsse redcngepp rkiipekns1 rqtynscarl  
 cinqetvclt  
 61 stamktencv akaklangts smivpkqrkl sasyekel cvkyfeqwe sdqvefvehl  
 121 isqmchyqhg hinsylkpm1 qrdfitalpa rglhdiaeni lsyldakslc aaelvckewy  
 181 rvtsgdmlwk kliermvrt d slwrglaerr gwgqylfknk ppdenappns fyralypkii  
 241 qdietiesnw rcgrhslqri hcrsetskgv yclqyddqki vsgrldntik iwdkstleck  
 301 riltghtgsv lclqydervi itgssdstvr vwdvnageml ntlihhceav lhlrfnngmm

SUBSTITUTE SHEET (RULE 26)

21/21

361 vtcskdrsia vwdmasptdi tlrrvlvghr aavnvdfdd kyivsasgdr tikvwntstc  
421 efvrtlnghk rgiaclqyrd rlvsgssdn tirlwdiecg aclrvleghe elvrcirfdn  
481 krivsgaydg kikvwdlmaa ldprapagtl clrtlvehsg rvfrlqfdef qivssshddt  
541 iliwdflndp aahaepprsp srtytyisr

SUBSTITUTE SHEET (RULE 26)

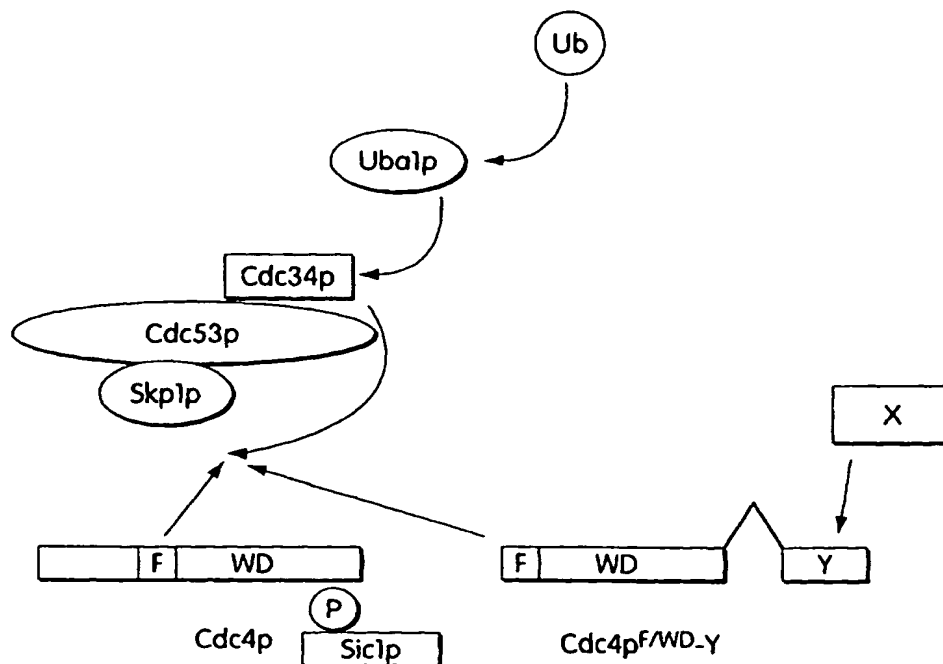
**THIS PAGE BLANK (uspto)**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/00, 15/62, 15/12, 15/37, 15/52, 5/10 // C07K 14/00</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/22110</b> <b>(43) International Publication Date:</b> 20 April 2000 (20.04.00)
<b>(21) International Application Number:</b> PCT/US99/23705 <b>(22) International Filing Date:</b> 8 October 1999 (08.10.99) <b>(30) Priority Data:</b> 60/103,787                      9 October 1998 (09.10.98)                      US <b>(71) Applicant:</b> PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). <b>(72) Inventors:</b> ZHOU, Pengbo; 34 Hammond Pond Parkway #3, Chestnut Hill, MA 02467 (US). HOWLEY, Peter; 5 Hundreds Circle, Wellesley, MA 02181 (US). <b>(74) Agents:</b> VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).	<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 16 November 2000 (16.11.00)	

**(54) Title:** TARGETED PROTEOLYSIS BY RECRUITMENT TO UBIQUITIN PROTEIN LIGASES



**(57) Abstract**

The present invention relates to methods and reagents for targeting proteolysis of a polypeptide by cis or trans association with a ubiquitin protein ligase, and further provides methods and reagents for inhibiting the ubiquitination and proteolysis of cellular proteins which are recognized by a ubiquitin protein ligase.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		



## INTERNATIONAL SEARCH REPORT

Intern 1al Application No  
PCT/US 99/23705

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/00 C12N15/62 C12N15/12 C12N15/37 C12N15/52  
C12N5/10 //C07K14/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATTON E.E. ET AL.: "Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis." TRENDS GENET., vol. 14, no. 6, June 1998 (1998-06), pages 236-243, XP002136980 the whole document --- -/--	1-13, 15-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

4 May 2000

Date of mailing of the international search report

09.08.00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Galli, I

## INTERNATIONAL SEARCH REPORT

Intern 1al Application No

PCT/US 99/23705

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SKOWYRA D. ET AL.: "F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex." CELL, vol. 91, no. 2, 17 October 1997 (1997-10-17), pages 209-219, XP002136981 cited in the application the whole document	1-13, 15-21
P,X	--- WO 99 18989 A (BAYLOR COLLEGE MEDICINE) 22 April 1999 (1999-04-22) the whole document	1-13, 15-21
P,X	--- ZHOU P. & HOWLEY P.M.: "Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases" MOLECULAR CELL, vol. 2, no. 5, November 1998 (1998-11), pages 571-580, XP002136982 cited in the application the whole document	22-25, 29-31
A	--- FELDMAN R.M. ET AL.: "A complex of Cdc4p, Skp1p, and Cdc53/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p." CELL, vol. 91, no. 2, 17 October 1997 (1997-10-17), pages 221-230, XP002136983 cited in the application the whole document	1-31
A	--- LI F N ET AL: "GRR1 OF SACCHAROMYCES CEREVISIAE IS CONNECTED TO THE UBIQUITIN PROTEOLYSIS MACHINERY THROUGH SKP1: COUPLING GLUCOSE SENSING TO GENE EXPRESSION AND THE CELL CYCLE" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 16, no. 18, 1997, pages 5629-5638, XP002915581 ISSN: 0261-4189 the whole document	1-31
A	--- US 5 252 466 A (CRONAN JR JOHN E) 12 October 1993 (1993-10-12) abstract -----	22-25, 29-31

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/23705

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9918989 A	22-04-1999	AU 1088399 A	03-05-1999
US 5252466 A	12-10-1993	AU 647025 B	17-03-1994
		AU 5827090 A	18-12-1990
		CA 2057908 A	20-11-1990
		EP 0472658 A	04-03-1992
		JP 4507341 T	24-12-1992
		WO 9014431 A	29-11-1990

16

THIS PAGE BLANK (USPTO)

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**